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Chemical and morphological investigations of the Ontogenetic effects of Novel Psychoactive Substances on forensically important blowfly species (Diptera: Calliphoridae).

A thesis submitted by

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to

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Abstract

Post Mortem Interval (PMI) estimation is a key concern of forensic entomology research. Numerous factors are recognized to affect these calculations, and have shown to potentially introduce error, leading to an incorrect time of death estimation. One such acknowledged factor is the presence of drugs and toxins. A range of Novel Psychoactive Substances (NPS), common adulterants and one illegal drug were tested on two blowfly species, of forensic importance, *Calliphora vicina* and *Lucilia sericata* (Diptera: Calliphoridae). An artificial diet was used to enable effective delivery of the substances; the effect on development was studied by observing larval length, weight, instar and time taken to reach pupariation. As a potential alternative for accurate aging, the cuticular hydrocarbon profile was analysed for changes in response to drug presence. Preliminary investigations were also carried out to detect the presence of NPS within larvae, by extraction and derivatisation. The NPS had a profound effect on the development of immature larval samples; rates were mostly accelerated, shown by larval length, weight and an increase in time taken to reach pupariation. A potential PMI overestimation of 48 hours was presented for a number of substances. Paracetamol was the only drug shown to produce developmental delay, up to 48 hours for a higher dose. The effects of drug dosage and potential to use data from chemically similar drugs for PMI estimations are presented; MDA is compared with NPS, 6APB. Noteworthy, the cuticular hydrocarbon profile showed no major changes in response to the drugs, some minor differences were observed but this was less pronounced than the development data and has lesser effect on PMI estimation. Results encourage the use of cuticular hydrocarbon analysis for accurately aging blowfly, despite showing developmental changes triggered by drug ingestion, which may otherwise cause incorrect PMI estimations.

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My PhD experience has provided some major challenges but if you do not challenge yourself, you will never realise what you can become. This thesis is presented to the reader as a result of impassioned research, throughout my time at Keele.

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Abbreviations

HC	Hydrocarbon
CHC	Cuticular Hydrocarbon
C. vomitoria	*Calliphora vomitoria*
C. vicina	*Calliphora vicina*
L. sericata	*Lucilia sericata*
PMI	Post-Mortem Interval
PMImin	Minimum Post-Mortem Interval
PMImax	Maximum Post-Mortem Interval
ADD	Accumulated Degree Days
ADH	Accumulated Degree Hours
GC-MS	Gas Chromatography - Mass Spectrometry
LC-MS	Liquid Chromatography - Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
HPLC-UV	High-Performance Liquid Chromatography, Ultraviolet–visible spectroscopy
TLC	Thin – layer Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
LOB	Limit of Blank
SP	Stationary Phase
MP	Mobile Phase

PCA	Principal Component Analysis
PCs	Principal Components
DMDS	Dimethyl Disulphide
LLE	Liquid-liquid extraction
SLE	Supported liquid extraction
APB	Amino propyl benzofuran
AMT	α-Methyl tryptamine
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine (Ecstasy)
6IT	6-(2-Aminopropyl)indole
5EAPB	1-(benzofuran-5-yl)-N-ethylpropan-2-amine
MPA	Methiopropamine
2AI	2-Aminoindane
NIST	National Institute of Standards and Technology
GSL	General sales list
ACMD	Advisory Council on the Misuse of Drugs
M/Z	Molecular Ion

1.1 Forensic entomology

Forensic entomology is the investigation of insects and other arthropods recovered from crime scenes and corpses, including the application of this knowledge to criminal investigations. The use of insects in connection with forensics goes back to the thirteenth century, although basic [1]–[3]. During the seventeenth century, the concept of metamorphosis was understood and explored, and it was in the nineteenth century that the connection was made between flies and corpses, but with no clear link to Post Mortem Interval (PMI). The first application of forensic entomology was in a French courtroom; in 1850, when the skeletonised remains of a child were found behind a chimney by workers during redecoration. Insect evidence was accepted as proof that the current occupants of the building could not have murdered the victim [4]. After this, insect succession on corpses was researched; followed by a wide variety of related topics such as the modification of corpses by insects. Forensic entomology may also help when dealing with cases of neglect; maggots can live in natural orifices or wounds of living people, the estimated age of these indicating the length of neglect [5]. Insect evidence can also be used to indicate post mortem movement of a body [6]. Increasingly providing useful results, entomology is now seen as an important part of a forensic investigation.

The extensive science of Forensic Entomology can be separated into three distinct areas; Urban, Stored-product and Medicolegal Forensic Entomology.

Urban forensic entomology concerns knowledge based on pest infestation relating to litigation. This is to include legal disputes relating to pests and extermination in the scope of houses and gardens. The use and misuse of pesticides is included here [4],[7]. Stored-product forensic entomology comprises of the investigation of insect infestation or contamination in relation to commercial products. The final category, medicolegal forensic entomology, will be the focus of this research project going forward. The study of insects and other arthropods and their connection at crime scenes would be classified in this sub-field of forensic entomology. Investigations surrounding death are a major focus.

1.1.1 Post Mortem Interval

Insects are comprised of about one million known species, found in every environment. Flies (Diptera) or more specifically blowfly (Calliphoridae) are good indicators of time since death as they are among the first colonizers of cadavers, often within only a few minutes dependant on the season, temperatures and body accessibility [4]. This is because they are attracted by the odour produced during decomposition [8], often from great distances [9]. The time which has elapsed since death (Post Mortem Interval or PMI) is crucial in forensic investigations as it can help to identify or eliminate suspects by focussing the timeframe and arguing or supporting an alibi [10],[11]**.** PMI can also have implications with a natural death for issues such as inheritance and insurance claims [12].

PMImin indicated using insects is known as the colonization time or period of insect activity [13]. Usually a pathologist would be able to indicate PMI accurately utilising the cadaver decomposition, up to 72 hours after death depending on various conditions, but

after this time a forensic entomologist is normally called [14]. Estimates of PMImin are generated reliably from standardised larval development curves resulting from research studies [15] under specific environmental conditions [16]. Variables that are known to contribute are also taken into account, for example temperature (both of the air and maggot masses), weather etc. Due to the number of variables, PMI is not given as a fixed value but as a maximum and minimum as shown in the figure below. For example, death of the body and arrival of the insects is not always simultaneous.

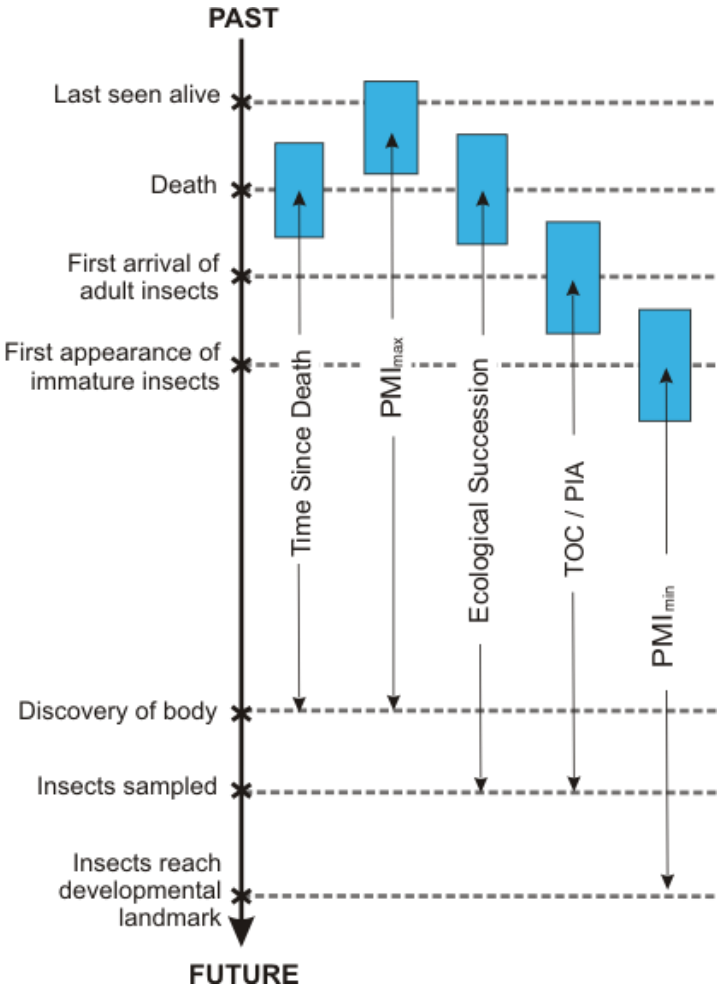


Figure 1: Relationships of duration of insect development (PMImin), time of colonisation (TOC), period of insect activity (PIA), ecological succession, maximum post-mortem interval (PMImax) and events surrounding a death. Redrawn and adapted from [13].

Correct species identification is the first step for PMImin estimation. Often this requires rearing the larval stages to adulthood as identification is difficult and usually requires an expert. Accurate identification is a priority as the most important implication for PMI estimation is that the insects differ in growth rate, arrival and order of succession [10].

Estimation of PMI is currently carried out using one of two main processes.

1.1.1.1 Minimum Post Mortem Interval (PMImin) estimated by insect development

During the initial stages of decomposition the extent of development of an individual species inhabiting a body is more useful for estimation of PMImin; the minimum time elapsed since death [7],[11],[17],[18]. Age determination of both the youngest and oldest stages is usually done by measuring larval length and comparing it with research data. Accurate estimation of PMI requires a detailed knowledge of the development of forensically important species under numerous conditions, including in the presence of drugs.

1.1.1.2 Minimum Post Mortem Interval (PMImin) estimated by insect succession

Insect succession information is utilised for estimation purposes when the individual in question is in the later stages of decomposition [7],[18]–[21]. As the corpse decomposes it provides a microhabitat to a selection of organisms. This habitat changes in chemical and biological composition as it reaches different stages of decomposition [21]–[26]. The

decomposing remains will attract different insects at different stages due to the conditions they offer as well as the changing temperature and humidity; as shown below in Figure 2. The order in which insects arrive on the body is known as the succession pattern as there will be continuous wave of larvae hatching [20]–[22], [24], [26]–[30]. Knowledge of and comparison to these patterns can help estimate PMImin by identifying the exact species present.

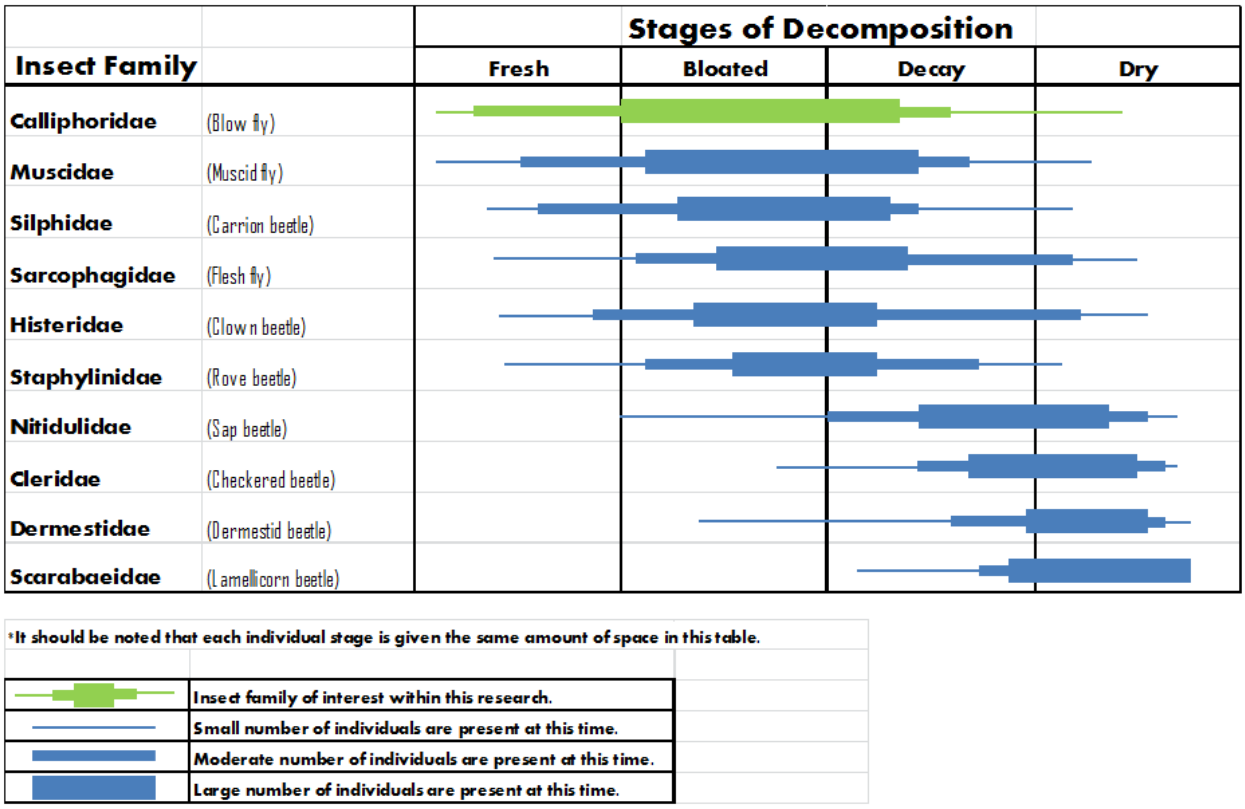


Figure 2: The succession pattern of insects on human decomposing remains, redrawn and adapted from [31].

1.1.2 Stages of decomposition

There are five main stages associated with classification of decomposing remains of a cadaver for educational purposes, decomposition is however a continuous process [3],[4],[32].

Fresh

The fresh stage initiates as soon as an individual is dead, and this continues through to blatant bloating of the body. Changes associated with the decomposition of this stage include discolouration of the abdomen and skin cracking. Blowflies are attracted to a cadaver at this stage. They both feed and lay their eggs, particularly around orifices or open wounds. Eggs laid during this stage also begin to hatch and feed internally; this is not always obvious superficially.

Bloat

Putrefaction initiates; Anaerobic bacteria which are found reproducing in the corpse cause an increase in gases and instigate the bloating stage; the cadaver has a balloon like appearance. Corpse temperature can increase providing an ideal habitat; the larval masses formed from eggs laid by blowfly also encourage this temperature increase. Larvae appear more visible, adult blowfly are still attracted to the body, Beetles can also be attracted to the cadaver at this stage.

Decay

Gases formed during the previous decomposition stages tend to be released through openings. These openings are caused by the presence of the colonisers and the increased larval masses. The corpse appears to deflate, and this marks the beginning of the decay stage. The number of beetles also increases at this stage, some feeding from the body,

others from the immature life stages of the blowfly. Some of the first insects to colonise the body start to leave in order to pupate. This stage of development is known as 'post feeding' [33]. Generally only skin and cartilage remain as this stage finalises.

Post decay

Mummified skin, hair, bones and cartilage remains. Most soft tissues have now been consumed by the earlier colonisers. Beetles still remain at this stage, Diptera are less prominent.

Skeletal

It is unlikely to find any insects colonising the body at this stage, only bones and hair remain. It is possible to note soil dwelling species such as mites.

1.1.3 Isomorphen and Isomegalen diagrams

Results from developmental studies can be used to produce Isomorphen and Isomegalen diagrams. These are useful tools for the estimation of PMImin with no need for related background knowledge and allow the development of insects to be easily visualised. Isomorphen diagrams (Figure 3) report the time from oviposition plotted against temperature and the expected morphological stage. These are especially useful when concerning post feeding larvae and pupae, when the larval length is no longer of interest. Isomegalen diagrams (Figure 4) are similar but show feeding larval stages and are based upon larval length [34].

Their use is limited in the field, due to variable temperatures causing incorrect interpretation. It is suggested that these graphs can be used to provide a minimum and

maximum age based on the known temperature ranges. No degree of accuracy is provided for these diagrams [18].

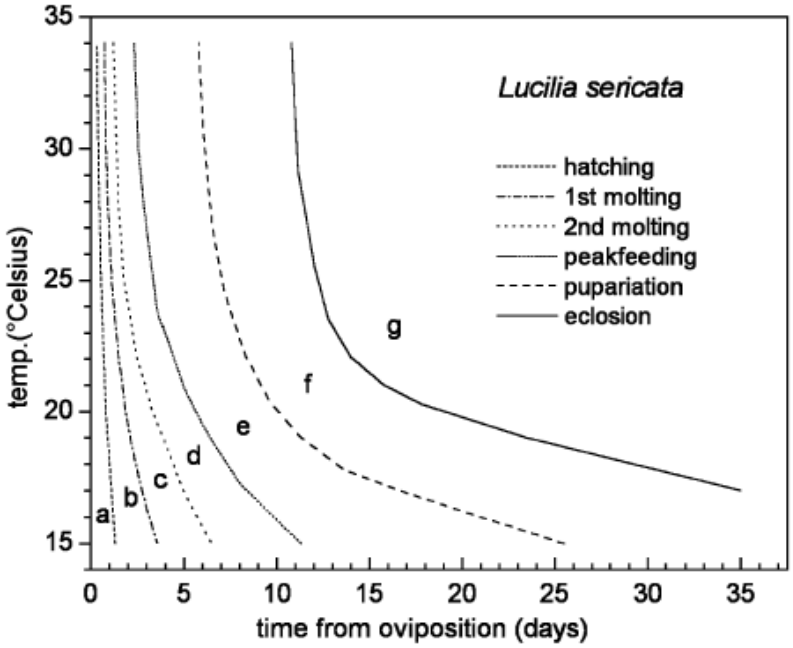


Figure 3: Isomorphen diagram of *Lucilia sericata* [34].

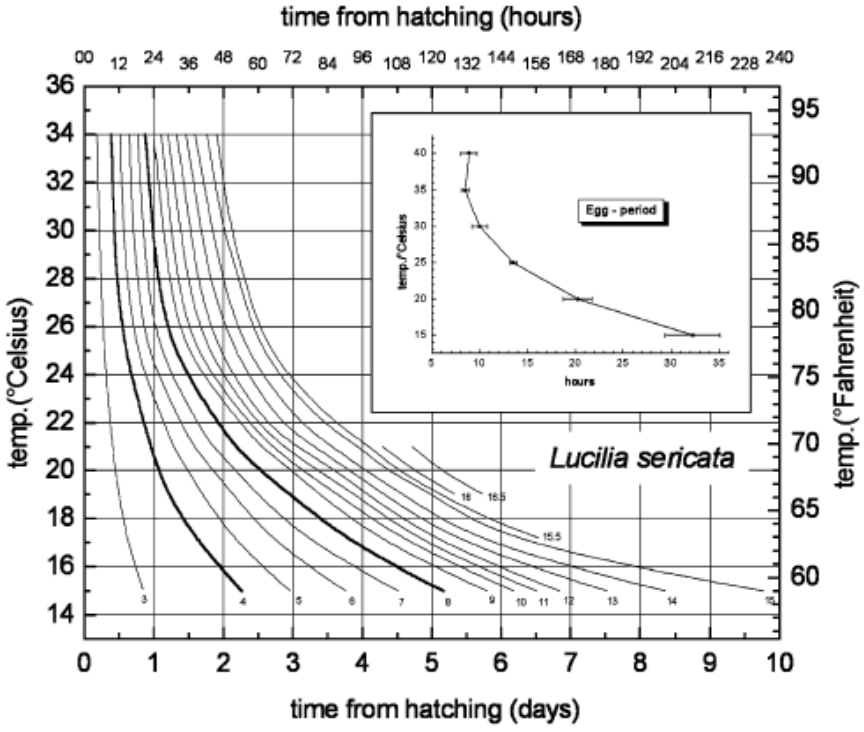


Figure 4: Isomegalen diagram of *Lucilia sericata* [34].

1.1.4 Thermal Summation models

Thermal summation modelling can also be used to represent development of insects. The Accumulated Degree Hours (ADH), or Accumulated Degree Days (ADD) is plotted against the larval growth, showing temperature dependant development rate. Accumulated Degree hours or days is a measurement of the thermal input required for the development of insects. Larval instar and measurements can be used to determine the time taken for the larva to reach the development stage in question [7],[35].

1.1.5 Lifecycle

Blowflies (Diptera: Calliphoridae) are recognised forensically as important tools for criminal investigations. This is due to their worldwide spread and predictable nature in an otherwise unpredictable environment.

They have adjusted to suit this, with a short life cycle and a large reproductive potential. Each female will lay around ten batches of eggs within the lifecycle with between 100 and 250 eggs in each batch.

For PMI estimation purposes, the six-stage lifecycle is well studied. There are three larval stages as shown in Figure 5 (1^{st}, 2^{nd} and 3^{rd} instar) where after they pupariate before emerging as adult flies.

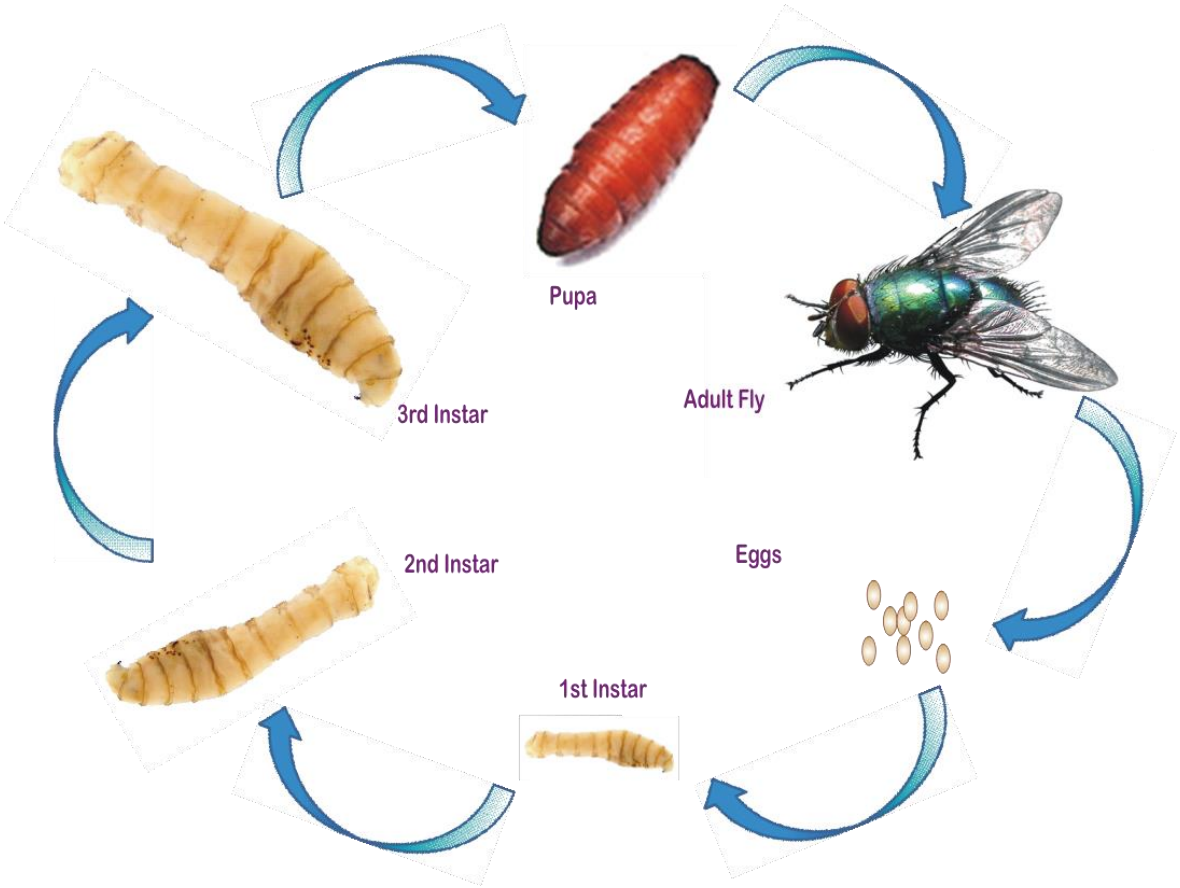


Figure 5: The lifecycle of a blowfly (Diptera: Calliphoridae).

1.1.5.1 Feeding to pupariation

Described here is the method used for locating the carrion by Calliphoridae, the system of feeding, development and eventual dispersion and pupariation.

Blowflies are a dominant species during the first stages of decomposition and often the first to arrive at a cadaver; this is within minutes if the conditions are suitable. *Lucilia sericata*, *Calliphora vicina* and *Calliphora vomitoria* are frequently amongst the first to arrive within the United Kingdom [11], [17], [22]–[24], [36]. Adult blowfly locate the carrion predominantly using their antenna, this is where sense organs are found [37].

All species mentioned are also readily available as a species inhabiting the United Kingdom and will therefore be the focus of the project going forward.

The female blowfly will commonly outnumber the male blowfly with around 8.8 females described for every male [38] Females are the most forensically useful as they create a timestamp by ovipositing.

Once they have arrived at the food source, the timing of oviposition depends on whether or not the female is gravid (with eggs). A female requires a protein meal in order for the ovaries to develop and they are then able to oviposit [39]. At this point females often lay near to one another producing egg masses [37]. Open orifices are the preferred oviposition areas, often including open wounds or regions such as eyes, ears, mouth, anus or vagina [22],[23],[40].

The timing concerned with hatching eggs will depend on the species of blowfly and the ambient temperature; as shown in the isomorphen and isomegalen diagrams above in Figure 3 and Figure 4, this is often between 6 and 40 hours [22].

Larvae are built for purpose with a sagittal anterior and blunt posterior which enables them to burrow into the carrion upon emergence from the egg [41]. This gives the larvae a form of protection from environmental conditions such as temperature, rainfall and desiccation which naturally vary [39].

Time spent at each life stage depends greatly upon environmental conditions and species, however, eggs are likely to hatch within 24 hours of being laid and the larval stages, on average, are completed within 5 days. This finalises with the hardening and darkening of the cuticle which forms the puparium.

Food sources favourable to these immature life forms are only available for a few days therefore, larvae must feed efficiently and progress through their larval stage quickly [42]

Larval digestion is comprised of two parts; external and internal. Firstly cadaver tissue is liquefied using the digestive enzymes externally secreted [43],[44].

Internal digestion involves hydrolysis and then absorbing the food, this is achieved using the movement of the pharynx and oesophagus [45].

Larvae progress through three different instars, as explained previously (Figure 5). The cuticle of the larvae is moulted as each instar is finalised, this allows growth. Once they have completed feeding in their third instar, they move away from the carrion before pupating. Some species can wander for a number of days before finding an area they consider best for pupariation and later pupation, often cool and dark [39] whilst others tend to pupate very close to the carrion. This is known as the post feeding stage.

After a suitable pupariation site has been selected, the two ends of the larvae then contract, and the cuticle becomes dark in colour and hardens. This casing provides protection during the metamorphosis from larvae to blowfly. This stage continues for around 7 days, species and temperature dependant, until eventually the fly pushes its way out using the ptilinum. The abdomen is dull in colour on emergence and the wings are shrivelled. It takes a few hours for the wings to expand and the blowfly colour to develop [41].

1.1.6 Factors affecting the lifecycle leading to incorrect PMI estimation

It is important when estimating PMImin to consider factors which may have an influence and in turn create estimation errors. If reference data is a closer match to the environment observed then the margin of error will be decreased [22], [10]. Biotic and abiotic factors, which affect insect activity, are a priority in current entomology research.

1.1.6.1 Weather, temperature and photo-period

Estimation of PMI is dependent on the temperature and environmental conditions. Temperature is known to affect insect metabolism and development rates, generally increasing with a rise in temperature [46]. Conditions found at the crime scene are crucial in the determination of development and succession; if conditions relating to the reference data are not very similar, then this may encourage error. Other areas for consideration include water drainage, and coverage. It is possible that long periods of heavy rainfall could wash away newly laid eggs [47]. Alternatively, rainfall could be seen to keep a food source moist and discourage it from drying out, this would encourage larval feeding [48]. It is possible that blowfly activity will be impeded in strong winds, rain and colder temperatures [49].

It is not temperature differences as compared to reference data alone that can cause error but also the fluctuation of temperatures. Temperature changes throughout the whole developmental period must be considered for a correct PMI estimation [18],[50].

Sunlight would also affect the temperature reached by the food source. The nature of the

ground and the contact with sunlight can vary the temperature reached considerably. The surroundings are likely to either absorb or radiate heat and direct or shaded sunlight can change the temperature by up to 11°C [39]. Certain species also prefer shaded or sunny areas, this can mean that the location of the cadaver can encourage different species dependant on their preference [51]–[53].

A final consideration in this area is the photoperiod experienced by the larvae and pupae alike. It is thought that the rate of development would be inhibited by constant light [54].

1.1.6.2 Genetic and geographic differences

Groups of the same species located in different geographical locations have been shown to have adapted to their local surroundings, causing genetic variation [55]. The differing conditions (for example: weather, temperature and elevation) produced in the locations can also help to explain the variation observed during development. The differences caused by this genetic variation have shown to be significant when estimating PMI using developmental data [55]. Variation naturally appears within a population also. Within one colony there will be individuals developing at differing rates. This can cause differing sizes for the same age [56].

The correct geographical population data must be used to discourage estimation error and improper interpretation [57].

1.1.6.3 Precocious egg development

Fertilised eggs and larvae can be present in the genital tract of the female blowfly [58]. At 22°C this can mean PMI estimations can be out by as much as 24 hours. If these larvae are collected as reflective of the population, this would cause overestimation of blowfly age.

1.1.6.4 Myiasis

Incorrect PMI estimations can be caused by Myiasis. Myiasis is the feeding by larvae on tissues, dead or alive, prior to death of the individual [18], [59]. This can cause an overestimation of time since death, potentially up to a number of weeks. Myiasis is predominantly seen in relation to the elderly or young children [5]. Blowfly can be attracted to the wound of a living person or preferred spots of colonization such as eyes, ear and nose and genital areas, especially during cases of neglect [5],[60].

1.1.6.5 Delays in oviposition and nocturnal oviposition

There may be a delay in the time taken for a blowfly to oviposit on a corpse [61]. Weather conditions could delay blowfly as discussed previously. The corpse could have been wrapped or buried in an attempt at concealment. This would restrict movement of blowfly both towards and away from the body. Some studies suggest the wrapping of a corpse could significantly delay penetration of the layer and eventual oviposition, some materials by as much as 7 days [62]. Other studies suggested that certain materials could actually assist oviposition due to increasing oviposition sites and shelter whilst retaining moisture essential to the body tissues [41],[63]. This is dependent on the material and the extent to which the cadaver is wrapped [64]. The state of dress of an individual must be considered, clothing itself can inhibit access and delay oviposition [27],[65]. The decomposition and pattern of succession is not only effected by burial of a cadaver but also the depth of the burial, some studies suggest the burying of a cadaver delays oviposition by a minimum period of 14 days [7], [8], [22], [66]–[68].

Interruptions could also be caused by a body located inside of a property or vehicle. Blowflies are still shown to oviposit but normally in lower numbers and delays of up to five days have been shown [69],[70]. Any possible delays must be considered for correct PMI estimation. Enclosed spaces have also been found to encourage ambient temperature to exceed the expected and increase developmental rate [16],[17].

Ground dwelling species would be prevented from colonizing a hanging cadaver due to inability to get to it and this would therefore alter the succession and likely also the decomposition rate [73].

It was previously believed that oviposition did not occur at night. Studies have since shown this to be untrue for the main blowflies of forensic interest, it does however result in a lower amount of eggs and there is reduced fly activity during nocturnal hours [18], [72]–[74].

1.1.6.6 Overcrowding and competition

Competition for food during development often causes larvae to develop quicker but will produce smaller larvae and in turn smaller than average adults, these are also likely to pupate quicker than normal [75], [76]. Different species of blowfly are affected differently depending on their ability to adapt to a competitive environment. Some larvae may disperse if competition level is too high and search for food elsewhere [18].

It should also be noted that when a large number of larvae are in the same location, the

temperature can be much higher than expected; this is known as a 'Maggot mass effect' [41]. The blowfly activity on a corpse alone can raise the temperature, having a detrimental effect on PMI estimation [77].

1.1.6.7 Other factors to consider, relating to decomposition rate

As explained previously, the stage of decomposition affects the insect species which are attracted; this is because of the differing conditions and the preferences of the individual insect species. If the rate of decomposition is changed in any way, then this will impact the succession of insects.

One study suggests that the burning of a cadaver [78] would change both the species composition and the decomposition process [59]. PMI would be underestimated if the succession pattern observed under normal conditions were applied to the carbonized remains [79]. Other research suggests that oviposition can occur between 24 hours and 4 days earlier on a burnt cadaver than un-burnt and all developmental stages following, will also happen prematurely [80].

Decomposition in fresh and marine water is significantly different. Characteristics typically associated with these sites include bloating, marbling, shedding of hair, scavenging, decay and exposure of internal organs, algae accumulation and algae staining on bones [76], [77], [79]. Water type, the temperature of the water and the season can all have an effect on the decomposition rate, as will depth and extent of submersion [81].

Scavenging is also known to alter the decomposition process due to the creation of wounds and the removal of clothing and other materials covering the cadaver [82]. Insects are the major colonizers of carrion whilst on land; they are attracted to wounds,

open lesions and natural orifices [83]. Studies [86],[87] have shown however; that in water, wounds were not as attractive in relation to animal scavenging; the animals tended to feed directly from the skin of the carrion and in turn created artefacts, which damaged the body and modified it. This can mean the loss of identifying features, but can also cause errors as post mortem artefacts may be confused for ante mortem injuries [84].

1.1.6.8 Body Tissue

Several studies have looked at the effect that different body tissues have on larval development and documented the variation [76],[86],[87]. One study found that larvae given pig tissue as opposed to cow tissue grew faster and resulted in larger blowfly adults. The study also suggested that the lung and heart tissue when compared with liver also increased growth [15]. It was also noted that when tissue composition was analysed by providing both liquidised and non-liquidised tissue; no difference in development was observed [15]. Another study found that when comparing heart tissue to brain, lung, kidney, intestine and minced meat that the larvae were much smaller and resulted in smaller adults [88]. It is therefore very important to consider the tissue type when interpreting PMI estimation and using developmental data, it should be as close to the scene environment as possible. It also highlights the importance of documenting the larval position during collection, as this should indicate the tissue type they have been feeding upon [76].

1.1.6.9 Drugs

Drugs and toxins are another consideration when determining PMI, as anything taken prior to death can be ingested by the larvae and lead to an overestimation or underestimation of PMI. Drugs are known to affect the development of blowfly. The reaction to different drugs varies from species to species [89]–[94]. This will be considered in greater detail later in this chapter in section 1.2.

1.1.6.10 Dispersion time

Once the post feeding stage is reached by the larvae, they will venture away from the corpse in search of a suitable pupariation site. The ideal pupariation site is a protected environment, cool and dark. For some larvae this site may take longer to reach, this consequently increases the time spent in this developmental stage and is unexpected when calculating PMI [95].

1.1.6.11 Pupae as contaminants

During a forensic investigation of insect evidence, the age of the oldest developmental life stage present must be calculated, in order to estimate the time since death of the individual. Blowfly spent the longest time during their development in the puparia stage. This stage is therefore forensically very important. However, as discussed previously, post-feeding larvae venture away from the carrion in search for a potential pupariation site. It is possible that they may pupate at a scene they did not originate from and mistakenly be counted as the oldest species present at a crime scene [18],[96].

1.1.6.12 Collecting, Killing and preserving

The collection, killing and preservation method can variably change the appearance and size of insect evidence. It must be noted where specimens are collected, including location on the body and environmental conditions. Specimens should be collected from everywhere on the corpse [97]. When killing larvae, certain solvents can cause putrefaction and shrinkage, making both PMI estimation as well as species determination from morphological characteristics, difficult [97]. Samples should be killed using hot, but not boiling water [97]. They should not be left in the water for longer than 30 seconds to avoid the breakdown of the cuticle. Incorrect killing of larvae can result in shrinkage and encourage error of up to 12 hours during PMI estimation [98].

Samples should be preserved in 70-95% ethanol [97]. Formalin and Formaldehyde, which are used during autopsies are known to shrink larvae quite drastically [39].

Samples taken for rearing should be kept under known temperature and other environmental conditions, if these are not controlled then PMI cannot be estimated without experimental error [97].

1.2 Entomotoxicology

1.2.1 Forensic Entomotoxicology

Forensic Entomotoxicology is a term which encompasses the two main areas within forensic research; the potential use of insects as alternative toxicological specimens and the effect of drugs and toxins on insect developmental time and Post Mortem Interval estimations. Both will be discussed in turn.

1.2.2 Insect use as alternative toxicological samples

Where conventional post mortem samples are not available for analysis, it has been demonstrated that Diptera can be reliable alternative specimens. The insight from these samples can help towards PMI estimation, possible movement of the corpse after death, possible anti-mortem injuries and the presence of drugs or toxins [99] .

Due to a rise in drug related deaths around the world [99], interest in this field has greatly increased. Detection of toxicological substances has been successful in larvae, pupae, puparial casing and Diptera adults whilst also in the faeces of beetles [100].

There are a number of possible reasons for utilising insects as toxicological samples; including situations where the cadaver is significantly decomposed, skeletonised or contaminated [101]. In some cases where the death occurred a large amount of time prior to discovering the body, the only abundant materials available are insect remains [93]. Research has shown that chemical substances can be detected in puparial cases,

which can remain unchanged for long periods after insect activity ceases [101],[102]and extends the potential useful timeframe for toxicological analysis into years [99],[103].

The absence of blood, urine or organs upon discovery and sampling of a cadaver would also require this alternative analysis [104].

Other possible reasons for use of insects as substitute samples include restrictions due to the religious beliefs of the victims' family; meaning no samples are permitted to be collected from the cadaver. It is also possible that biological traits could be altered due to the circumstances connected with the death [101].

In 1992 the first forensic case was recorded where drugs were detected in blow fly larvae leading to cause of death determination. The body had decomposed; samples from the muscle, along with insect larvae were analysed. The analysis confirmed the presence of cocaine and a metabolite associated with the breakdown of this drug. This information helped lead to the establishment of a cocaine overdose causing death [105].

Larvae feeding on tissues from an individual who had taken drugs prior to death are likely to be ingesting both the substance and its metabolites [94]. A drug can only be detected in larvae when the rate of absorption by the larva feeding, exceeds the rate of elimination from the body of the larva. Research has confirmed the accumulation of drugs inside feeding larvae using immunohistochemical techniques [93]. Elimination of drugs however, means the absence of drugs and toxins from most third instar larvae and pupae, unless a constant supply of toxins is provided for consumption [99].Future work is likely to focus on bioaccumulation and elimination from the larvae and how this effects the development [91]. Many compounds have been detected in insect tissues in a forensic

context; it is a heavily researched field. Table 1 outlines current research involving drugs and toxins associated with insect specimens. Referring to the table; Exuvia is defined as the cast-off outer skin of an arthropod after a moult, while Frass is the excrement of insects. The term Crop is used to refer to the part of the alimentary tract used for the storage of food.

Table 1: Summary of research involving different drugs and toxins

Drug /toxin	**Insect taxa**	**Developmental stage**	**Reference**
Ethanol	Calliphoridae, Sarcophagidae, *Phormia Regina.*	Larval	[89],[106]
Nicotine	*Calliphora vomitoria*	Larval	[107],[108]
Paracetamol	*Chrysomya rufifacies*	Larval	[109]
Arsenic trioxide	*Sarcophaga bullata*	Larval	[110]
Heroin	*Lucilia cuprina*	Larval	[111]
Ketamine	*Lucilia sericata*	Larval	[112]
Methamphetamine	*Calliphora stygia, Calliphora vomitoria*	Larval	[112],[113], [114]
Amitriptyline	*Lucilia sericata, Calliphora vicina, Dermestes maculatus, Megaselia scalaris.*	Larval, Pupae, Puparia, Exuvia, Frass and Crop.	[100],[115],[94] ,[116],[117],[118],[119]
Clomipramine	*Lucilia sericata*	Larval	[115], [94],[120]
Dothiepin	Not specified	Larval	[115]
Fluoxetine	Not specified	Larval	[115]
Nortriptyline	*Lucilia sericata,*	Larval. Puparia,	[100],[94]

	Dermestes maculatus, Megaselia scalaris.	Exuvia, Frass.	
Trazodone	*Calliphora vicina*	Larval	[119]
Trimipramine	*Calliphora vicina*	Larval	[119]
Venlafaxine	Not specified	Larval	[115]
Amobarbital	Not specified	Larval	[115]
Barbiturates	Not specified	Larval	[115]
Phenobarbital	*Cochliomyia macellaria, Lucilia sericata*	Larval	[104],[107],[94], [113]
Secobarbital	Not specified	Larval	[121]
Sodium amylobarbitone	*Calliphora vicina*	Larval, Pupae.	[122]
Sodium barbitone	*Calliphora vicina*	Larval, Pupae.	[122]
Sodium brallobarbitone	*Calliphora vicina*	Larval, Pupae.	[122]
Sodium phenobarbitone	*Calliphora vicina*	Larval, Pupae.	[122]
Sodium thiopentone	*Calliphora vicina*	Larval, Pupae.	[122]
Alprazolam	*Calliphora vicina*	Larval, Pupae.	[115],[123]
Bromazepam	*Piophila casei*	Larval, Pupae, Adult	[115],[124]
Clonazepam	*Calliphora vicina*	Larval, Pupae, Adult	[123]
Diazepam	*Calliphora vicina, Chrysomya albiceps, Chrysomya putoria*	Larval, Pupae, Adult	[102],[123]
Flunitrazepam	*Calliphora vicina*	Larval, Pupae, Adult	[123],[125]

Lorazepam	*Calliphora vicina, Calliphora loewi*	Larval, Pupae, Adult	[112],[115],[123]
Nordiazepam	*Calliphora vicina*	Larval, Pupae, Adult	[115],[123]
Oxazepam	*Calliphora vicina*	Larval, Pupae, Adult	[115],[120],[123]
Prazepam	*Calliphora vicina*	Larval, Pupae, Adult	[102]
Temazepam	*Calliphora vicina*	Larval, Pupae, Adult	[119],[123]
Triazolam	*Calliphora vicina*	Larval, Pupae, Adult	[115],[120],[123],[126]
Amphetamine	*Calliphora vicina*	Larval	[106],[122]
Benzoylecgonine	Not specified	Larval	[105]
Cocaine	*Lucilia sericata, Chrysomya albiceps, Chrysomya putoria*	Larval	[105],[94],[127],[128]
Digoxin	Not specified	Larval	[115]
Meprobamate	Not specified	Larval	[115]
Nefopam	Not specified	Larval	[115]
Sodium Aminohippurate	*Calliphora vicina*	Larval	[122]
Sodium salicylates	*Calliphora vicina*	Larval	[122]
THC-COOH	Not specified	Larval	[115]
11-Hydroxy-THC	Not specified	Larval	[115]
Codeine	*Lucilia sericata, Chrysomya rufifacies*	Larval, Pupae, Adult	[115],[129],[130],[131],[132]
Methadone	*Lucilia sericata*	Larval	[133],[134]
Morphine	*Dermestes freshi,*	Larval, Pupae, Adult, Puparia	[93], [131],[135],[136

	Thanatophilus sinuatus, Lucilia sericata, Calliphora stygia, Calliphora vicina, Protophormia terraenovae		],[137],[138],[139],
Opiates	*Lucilia sericata*	Larval	[94],[130]
Pholcodine	Not specified	Larval	[115]
Propoxyphene	*Calliphora vicina*	Larval, Crop	[115],[116]
Alimezanine	Not specified	Larval	[115],[120]
Chlorpromazine	Not specified	Larval	[115]
Cyamezanine	Not specified	Larval	[115]
Levomepromazine	*Lucilia sericata, Piophila casei*	Larval	[115],[94] ,[124]
Thioridazine	*Lucilia sericata*	Larval	[94]
Antimony	*Calliphora dubia, Lucilia sericata*	Larval, Pupae, Puparia, Adult.	[140],[141]
Barium	*Calliphora dubia, Lucilia sericata*	Larval, Pupae, Puparia, Adult.	[140],[141]
Cadmium	*Lucilia sericata*	Larval, Puparia, Adult.	[142]
Lead	*Calliphora dubia, Lucilia sericata*	Larval	[140],[141]
Mercury	Calliphoridae	Larval, Puparia, Adult.	[143]
Thallium	Calliphoridae	Larval	[144]
Malathion	*Chrysomya megacephala, Chrysomya rufifacies*	Larval, Pupae	[125]
Parathion	Diptera	Adult	[125]

To enable the analysis of insects as alternative toxicological samples; the drugs must be extracted from them. There are a number of methods shown in the literature; toxicological analysis for insect material is often the same as that used for human tissue and body fluid samples, including the use of GC-MS, GC and HPLC [91],[145],[146].

Some samples are homogenized with a solvent [115], or digested using a strong acid or enzyme [140] and others were crushed using a grinder [129],[130]. Gosselin et al suggests homogenization as the best method for breaking down a sample and avoiding cross contamination with other samples [92]. Tracqui et al suggests samples are then extracted using solid or liquid phase extraction procedures then analysed by GC-MS/ LC-MS depending on the drug [115]. Hedouin et al [147] measured concentrations of morphine using radioimmunoassay techniques while Introna et al measured opiate concentrations using the same detection method [130].

A review paper by Gosselin et al showed that the majority of samples are extracted with either liquid-liquid extraction (LLE) or supported liquid extraction (SLE) and then analysed using GC-MS, LC-MS, HPLC or HPLC-UV [92].

Bourel et al reared a colony on portions of minced meat spiked with morphine, specimens were sampled by homogenising then centrifuging, the supernatant was then analysed for morphine content. Morphine was detected, but only in the cuticle of the insects reared on concentrations of 100 and 1000 mg/kg. Higher concentrations were detected in the puparial cases [93]. The majority of research at present has been unable to quantify drugs and toxins accurately, a topic that will be discussed further, later in the chapter.

1.2.3 Effect on blowfly developmental data

The effect of the toxins or drugs on the growth and development of the blowfly is also of great research interest, as it can affect PMI estimations [91],[93]. Different species appear to have different responses to drugs and the rate in which they develop can increase, decrease or stay the same [148]. The reaction of the species to the presence of a drug may not always mirror that experienced by the human user, for example; stimulants may not increase activity, just as depressants may not reduce it.

Incorrect PMI estimation can be caused by the larva physically looking like a different developmental stage to that expected considering their time since emergence.

One case concerning developmental changes observed in blowfly larvae; describes the body of a young woman in early stages of decomposition. Insect colonization was focussed around the upper torso and the face. The majority of the larvae collected were 6-9mm in length, which indicated a PMI of 7 days. One larva was witnessed at a length of 17.7mm; this would give a PMI estimation of 21 days. It was subsequently shown that the individual larva in question was found around the nasal passage and that the woman had snorted cocaine prior to her death. During feeding the larva had ingested the cocaine and its growth was accelerated dramatically, causing a developmental difference of 14 days in terms of PMI [91],[149]. Another study showed the effect of larvae feeding on livers taken from rabbits which had been injected with cocaine. Those larvae feeding on the spiked liver developed faster than those feeding on control liver, which had not been spiked. The study concluded that cocaine had indeed influenced and stimulated larval growth. Should the presence of the drug not have been taken into account then this

would introduce a large PMI estimation error [150]. Other papers have also shown drugs and toxins interfere with usual larval development and consequently require extended research to determine correct developmental data considering these conditions [140], [151], [152]. Sometimes just the presence of the toxin will affect the insect but in other circumstances it will need to be present in a certain concentration before having any influence upon development [99]. Goff and co-workers have conducted a number of studies with a variety of drugs in which he administered the chemical to a live animal [90],[117],[145],[153],[154]. This type of study is useful because it can provide concentration data for both drugs and their metabolites relating to particular dosages administered.

1.2.4 Limitations of Entomotoxicology

The large amount of research carried out in the field of Entomotoxicology has brought about an expanse of new information, including a number of limitations within the field.

1.2.4.1 Drug Elimination

One such limitation was shown by Sadler et al [119], where research revealed that the absence of a drug from the larval sample was not necessarily indicative that the drug was absent within the food source, possibly due to drug elimination. It is expected that there are elimination mechanisms in place within the insect, since it has been shown in previous research that drugs do not bio-accumulate through the life stages unless there is constant exposure to the drugs and toxins [119]. It is essential to determine which insect phase would be most valuable for the detection of drugs for entomotoxicologists.

1.2.4.2 Insect Sampling

Drug concentrations are also known to decrease when larvae are taken from feeding on a spiked food source to drug free food source, which highlights the importance that larvae collected, must be sourced from those insects actively feeding from the corpse [119].

Insect sampling is seen as a simple part of the analysis procedure but it is a factor which can cause high variability in drug detection [92]. Sampling site, sampling variation, specimen numbers and accurate and clear sampling details are all variables which must be considered, this is on top of the common protocols for sampling collection relating to all insect evidence, for example; the killing, storage and decontamination of the insect specimens.

1.2.4.3 Metabolism

Artificial food sources or organs which are spiked with drugs after death are often used in this area of research, due to ethical concerns and also the inability to make use of drugged human tissue due to the Human Tissue Act, with obvious drawbacks [155].

The most apparent drawback concerning the imitation of expected scenarios is metabolism. Metabolism is a sequence of chemical reactions inside a living cell. Drugs which are injected into an organ after death or added to an artificial food source are not metabolised. This means that drug availability within these samples will not necessarily mimic that of a real scenario, metabolites of the original drug will also not be produced [156]. To get past this limitation requires the use of live animals which will be injected whilst still alive, time given to allow the drug to metabolise and then killed for analysis; unfortunately, this can create ethical complications and requires ethical approval which can be a lengthy process.

Metabolism is an important consideration in the analysis of drugs in insects. In a real-life scenario, the drugs will usually have entered the body before death and therefore it is likely that they will have been at least partially metabolised. It needs to be considered that the metabolites of the drug may also be present in the insect sample. The insect itself may also metabolise the drugs or even the metabolites. It is known that the pharmacokinetics of drugs in insects depends on the species, the developmental stage and the feeding activity [92], [129]. According to Parry et al [139] drug metabolism in insects occurs in the Malpighian tubules via cytochrome P450 and glutathione transferase enzymes. However, drug metabolism in insects is not yet interpreted. Presence of metabolites could result from the action of substrate enzymes [129] or larval metabolism [151]. Metabolism and drug excretion still requires a large amount of research; this will in turn increase the reliability of the results from this field within forensic science.

1.2.4.4 Developmental changes from alternate causes

There may also be other reasons separate from the effect of drugs, which can change the expected development of the insects concerned, for example competition. Competition is defined as a large number of insects with not sufficient amounts of food source, therefore creating competition for the available food, insects may not get the nutrients needed to develop at the expected rate [157], further information on this topic can be found previously in section 1.1.6.

It is important to note that this field relies on the recreation of the exact conditions found at the scene, such as humidity, temperature and photoperiod. These conditions can alter the lifecycle or drug concentrations significantly and therefore must be thoroughly researched [4].

1.2.4.5 Quantification

At this point research and reasoning suggests that the exact concentration of a particular drug found in an insect is not relatable back to the concentration in the human tissues. Interpretation of something dependant on so many variables has its limitations [115].

Comparisons of the drug concentrations between human or animal tissue and blowfly larvae show a varied pattern of distribution [94]. On one level, these differences could be attributed to the variances in physiology between the species or perhaps the chemical properties of the specific drug and how it interacts [94].

There have been many studies to confirm the quantitative analysis of drugs from within blowfly life stages, the issue regarding quantification arises when the intention is to extrapolate such data to infer human tissue concentration [94]. This is unreliable because of many uncontrollable variances.

When concentrations observed in larval or adult specimens are lower than those of the blood or tissue, it can be explained through metabolism and elimination by the insect. The stage of progression of the insect, through its lifecycle can profoundly affect the concentration detected. Larvae which are actively feeding, store ingested food in their crop; this expands during periods of ingestion. At this point the drug would be concentrated within the crop and it could be perceived that a larger concentration is present. The crop is also known to empty very quickly upon conclusion of feeding [41], this would potentially result in no drug detected within the insect sample. Absence of chemicals from the larvae therefore does not definitively indicate absence from the food source [118].

The region of the food source, which the larvae will feed upon, will also determine the concentration of the drug found there, due to the distribution of the drugs around the body. Larvae are known to wander on a cadaver and therefore the exact locations of feeding may not be known or may change. Concerning laboratory studies; The type of animal model used will have an effect on drug availability as distribution and metabolism is likely to differ [158] and it will not perfectly simulate the human model [156]. If larger animals are utilised to mimic the human model, as closely as possible, such as pigs, instead of smaller animals, such as rabbits and rats then replications are not as easy [89] and ethical problems arise. One study found that samples which were not washed before analysis showed significantly higher concentrations compared with those samples which were washed correctly [119], this shows that for successful quantitative analysis, at any level, a washing protocol should be followed [130].

The usefulness of insects as alternative toxicological specimens must be emphasized, even if the data is only qualitative. A positive identification of a drug, through insect analysis can confirm and support a diagnosis of intoxication [159].

1.3 Novel Psychoactive Substances

As seen from previous research, emphasis throughout has remained on controlled drugs, this is understandable due to the forensic relevance and the connection with death rate.

However, 204 deaths in 2015 were linked to Novel Psychoactive substances (NPS) in the UK [160]. There has been an increasing trend in the number of designer pharmaceutical and herbal drugs, often sold as 'legal' alternatives to illicit drugs [161]. There has been an upward trend in hospital admissions due to the toxicity and unknown or unreliable contents with unpredictable effects [162].

Currently there are 640 varieties of known Novel Psychoactive substances (NPS) globally, more commonly known as 'legal highs' [160], although some see this as an inappropriate term due to the misleading assumption that these chemicals are still uncontrolled [163] . These substances often mimic the effects of the traditional illegal drugs but are frequently stronger, markedly cheaper and potentially more dangerous due to the unknown ingredients.

The prevalence of these drugs has caused significant concern across the United Kingdom [162], northern Europe [164],[165], Australia [166], New Zealand, United States of America and globally [167]. This emergence of new psychoactive substances has raised prominent challenges in the field of drug policies and drug addiction [168].

NPS's tend to fall broadly under four categories [162]:

1) Named and specific substances which are chemically very similar to controlled drugs, but are structurally different enough to avoid being classified as illegal substances under the Misuse of Drugs Act, see
2) Figure 6 for an example concerning benzofurans.
3) Herbal and fungal substances and the extracts of these.
4) Products with names but no indication of ingredients.
5) Substances related to medicines.

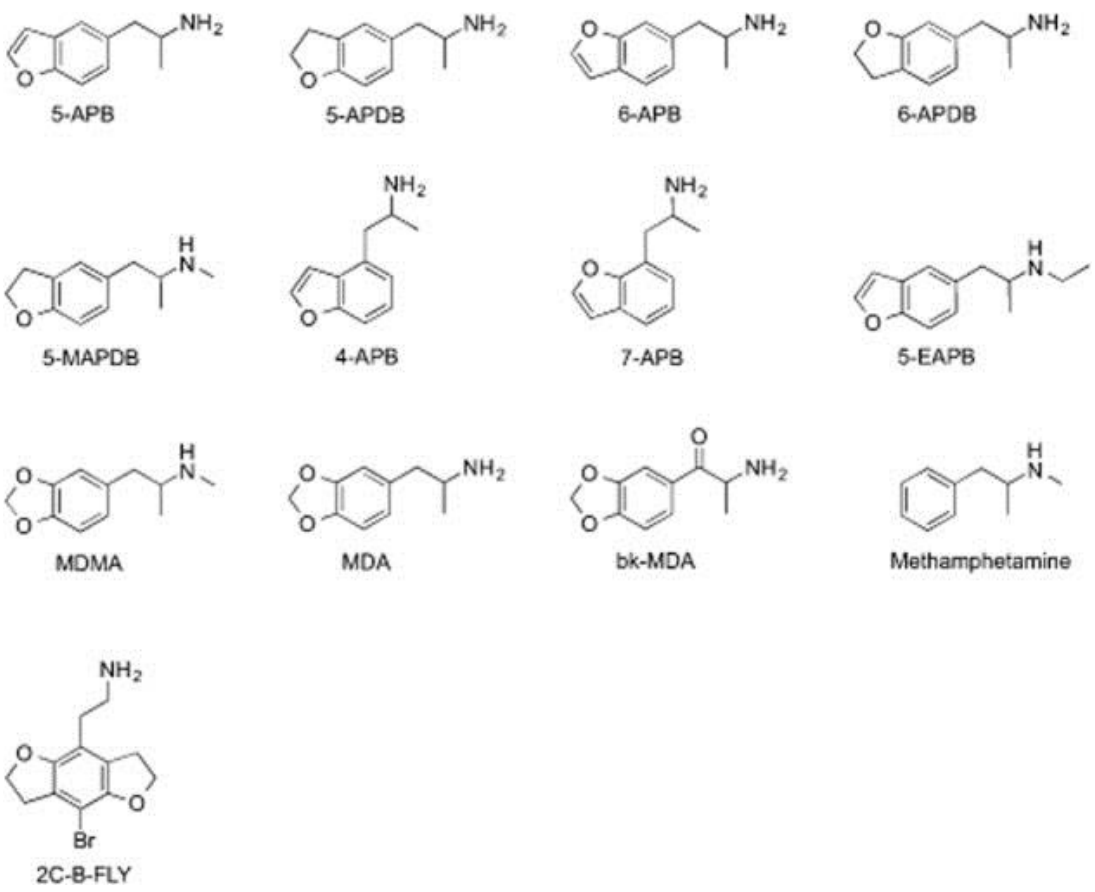


Figure 6: Chemical structures of benzofurans and related amphetamines [169].

Britain had the largest NPS market of any European country [160] and the most online headshops (Figure 7). Record numbers of new substances were detected across Europe in 2011 [170], with figures showing that a new legal high was appearing every week [171]. Very little is known about the effects of these drugs, especially in the long term, due to lack of research.

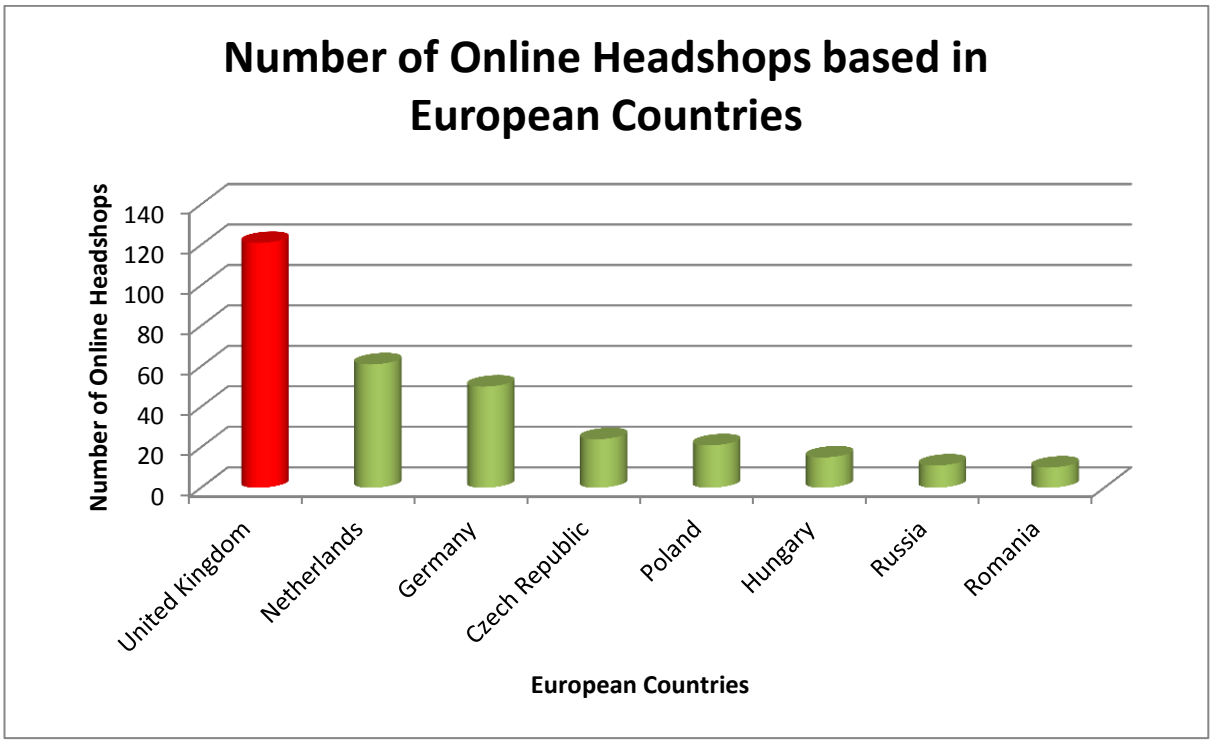


Figure 7: Numbers of online head shops based in European countries, adapted from [172] .

These substances first appeared in 1772 with the discovery of nitrous oxide (laughing gas) which then saw a revival in the 21st century [162]. NPS are therefore not recent but have, in the last few years become prominent due to a number of factors; advancement in chemical technologies, market availability, internet supply, trends in substance misuse and of course, price [162]. A British crime survey estimates that around 12 million individuals between the ages of 18 and 59 have used illicit drugs. Over recent years a shift has been seen in the range and type of drug use, with an increase in the use of NPS [173].

It is suggested that half a million 18-24 year olds have experimented with NPS at least once [160]. Research looking at pooled urine samples from urinals in London detected 6 potential NPS metabolites across all urinals surveyed [173]. Deaths have tripled in the last few years, predominantly in the younger generation [160]. An online survey by 'Mixmag', before these substances were banned which targeted British clubbers; found that 56.6% of respondents claimed to have used NPS. 20% of the respondents said that they had bought legal highs or 'research drugs' from the internet and stated that their primary reason for purchasing these substances was the due to the ease of being able to purchase them online or from a shop [174]. It has been hypothesized that NPS are often used as 'gateway drugs' as an introduction into illicit drug use [175].

There are different types of legal highs; stimulants, psychedelics, dissociatives, deliriants and depressants. Many are thought to be synthetic amphetamines, which give a psychoactive effect. They will therefore share many of the adverse effects and dependence liability issues of controlled amphetamines [162]. This includes high risks of overdose, acute toxicity and harm. The RedNet (Recreational drugs European network) project, which ran between 2010 and 2012, aimed to improve our knowledge about these drugs. A number of these substances were analysed, and the results are shown in Table 2. Over 33% were found to be 'spice' type synthetic cannabis drugs, a drug which studies suggest leaves users 30 times more likely to end up in the emergency department than conventional cannabis users [176], 27.5% MDMA 'type' drugs (Phenethylamines) and 19% psychedelic type Phenethylamines.

Table 2: Analysis of NPS and the main substances identified during the RedNet project between 2010-2012 [168].

Class	Main substance	Number
Phenethylamines	MDMA-like drugs	179
Synthetic cannabimimetics	Spice drugs	220
Cathinones	Mephedrone, MDPV	30
Tryptamine	5-Meo-DALT	69
Phenethylamines	5-APB, 6-APB	126
GHB-like	GBL, 1,4-BD	3
PCP-like	PCE, MXE	5
Piperazine	MBZP	2
Herbal plants	Salvia divinorum	6
Medicines	Pregabalin	10

One report also showed that 19% of seized samples analysed actually contained an already controlled substance [162]. Ivory wave, a NPS , is expected to contain 2-DPMP but one study actually observed a banned cathinone in some samples [162]. Sampling of random legal highs by one research team concluded that the majority of active ingredients were piperazines followed by cathinones [177].

It should also be noted that NPS content was inconsistent and unreliable. Products were altered so often that they could not often be analysed month to month, as the same product did not exist [177], [162].

Impurities and 'packing' ingredients are as much of a worry with NPS as with conventional illicit drugs. Caffeine is a major impurity found in these substances; it produces a psycho stimulant effect and therefore still provides an effect on the user. One study examined numerous samples and determined the concentration of caffeine to be significant in over 60% of samples surveyed, some of these with up to 96% caffeine content [168]. These types of substances have their own inherent toxicity that can be additive to the toxicity of the NPS. There is significant risk for individuals using these NPS of caffeine toxicity [178]. Data from the analysis of seven NPS in relation to caffeine content is shown in Table 3.

Table 3: Caffeine content of 7 NPS [178]. The quantitative analysis of the taurine content was approximate and accounts for why the total exceeds 100% for some products.

Product	Website	Caffeine content	Approximate maximum total caffeine dose	Taurine content
Blowout	EveryoneDoesIt	87%	870 mg	0%
Snow Blow	EveryoneDoesIt	94%	940 mg	0%
Sn* Berry	Submitted by UK police force	96%	960 mg	0%
NRG2	EuphoriaPlantFood	2%	20 mg	99%
DMC	EuphoriaPlantFood	48%	48 mg	54%
Synthacaine	EuphoriaPlantFood	94%	94 mg	0%
Ivory Wave	EuphoriaPlantFood	44%	44 mg	63%

Many suppliers use descriptions such as bath salts, incense or plant food. By claiming that the substances 'are not intended for human consumption' they were able to get around the outdated drug laws [179].

The presence of NPS is often overlooked in coronary reports; in 2010, a 24-year-old chef had bought a pack of Ivory Wave online on the Isle of Wight. Apparently, the drug produces similar effects as ecstasy. His body was found after he had been spotted running along a cliff. Whilst he did have alcohol and Ivory Wave traces in his bloodstream, the post-mortem ruled his death as one from a brain injury caused by falling from the cliff, after believing he could fly [172].

Brief analysis using Google trends, a public web facility based on google searches; shown in Figure 8, revealed a perverse link between the media's coverage of deaths due to legal highs, and the number of people searching online for ways of buying them. The top search term relating to legal highs in the United Kingdom is 'buy legal high'.

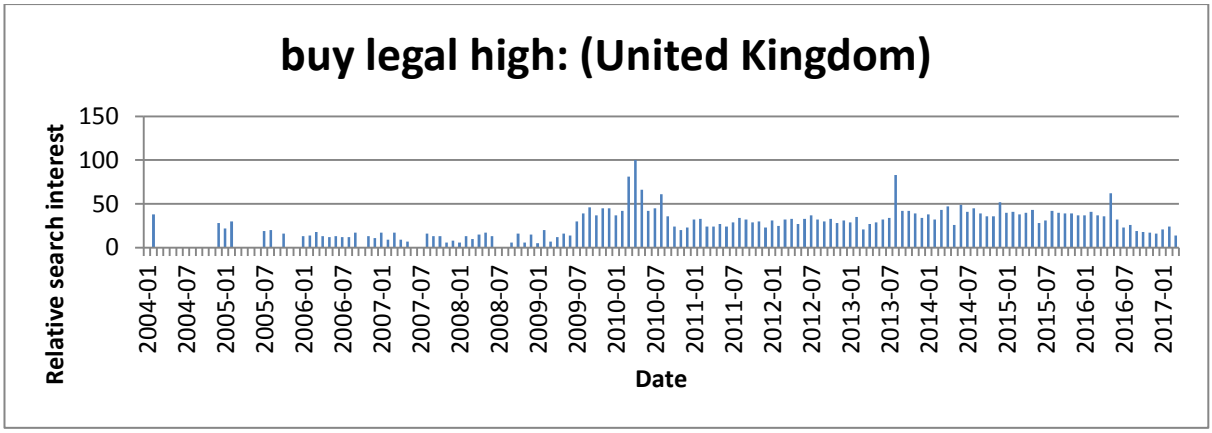


Figure 8: Popularity of google search term 'buy legal high' from 2004 until 2017 [180].

The government became extremely concerned about the increasing popularity of legal high drugs and new powers were introduced enabling legal highs to be banned temporarily for 12 months whilst they were researched [181]. In April 2016 the government in the UK passed a law banning the manufacture, supply and sale of NPS in an attempt to control a volatile situation [182]. The act intended to remove any semi 'legitimate' sales of these substances, online and in head shops. Because of the trend in the NPS industry of ever-changing analogues, the compounds are controlled based upon their psycho-activity. The act bans any substance that is likely to be used for its psychoactive effect and is not dependent on the potential harm it may cause. Some items are exempt from the act, such as; nicotine, alcohol, prescribed medical products and anything already covered by the Misuse of Drugs Act 1971.

The definition of 'Psychoactive' is a bone of contention since the commencement of the act. The act simply defines it as "any substance which (a) is capable of producing a psychoactive effect in a person who consumes it, and (b) is not an exempted substance" [182]. The government are content that this can be tested and proven in court, but a number of organisations claim this to be too broad of a term. The substance must be

shown to have a psychoactive effect on the individual before prosecution and there is doubt that this is realistic [160]. There have been over 500 arrest nationwide in connection to NPS but only a handful of convictions [160]. It is of common belief that the act was passed in a rush in an attempt to combat a growing issue but an act with so many areas of ambiguity and uncertainty is causing issues, which require clarification.

In applying such legislation to this problem, unintended consequences must be considered, such as; the emergence of a criminal market to fulfil supply and the potential introduction of adulterants, as often seen with illicit compounds [162].

1.4 Potential new methods and Cuticular Hydrocarbons as a tool to study development

Difficulties encountered during the identification process when using taxonomic keys have led to the application of other disciplines, to encourage progression and understanding. One such area is genomic analysis. Certain techniques can provide information such as geographical origin and the evolution of a species [183], [184] as well as markers to enable differentiation between blowfly species [6], [185], [186].

Previous research has shown encouraging data for aging and identifying insects using hydrocarbon profile analysis. Studies using Diptera have shown hydrocarbons can reliably be used for taxonomic purposes and a potential aging tool [181]–[185]. It is possible that hydrocarbon profiles may not be affected by drugs and toxins and therefore may prove an invaluable method for accurately aging larvae affected by such chemicals.

Cuticular hydrocarbons are found in the cuticle of every insect in all life stages and are very stable. The cuticle of an insect is a layer containing hydrocarbons, fatty acids, waxes, alcohols, glycerides, phospholipids and glycolipids [187], this layer prevents the desiccation and water penetration of the insect [188].

These compounds consist only of hydrogen and carbon, as suggested by the name and are held together in a long carbon chain; in insects usually approximately 19 to 35 carbon atoms long [187], although this may portray a limitation of the analytical technique as other studies have revealed hydrocarbon chains as long as 70 carbon atoms [189], [190].

It is possible for hydrocarbons to be saturated or unsaturated. Saturated hydrocarbons are known as n-alkanes or paraffins; all of the carbon atoms are joined with single bonds and one or more methyl groups may be present [187],[191],[192]. Unsaturated hydrocarbons are named according to the number of double bonds present along the length; One double bond forms an alkene, two an alkadiene and three an alkatriene [187], see Figure 9. These are also able to form two isomers known as Z and E alkenes [187]. It is known that chain length and the presence of methyl-groups along with double bonds impacts the physical properties of the hydrocarbons [193].

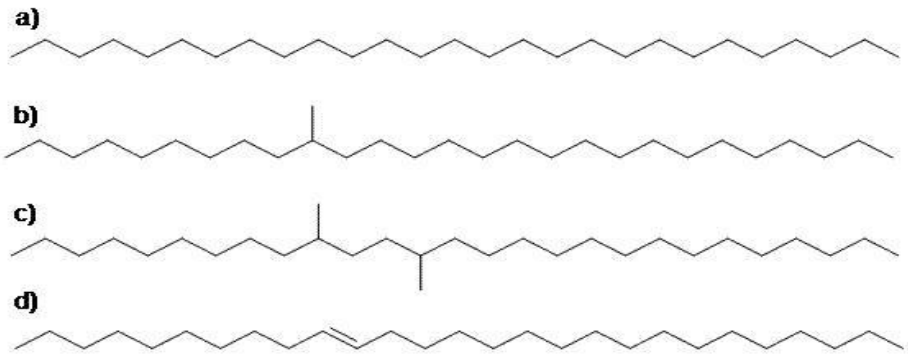


Figure 9: a) Straight chain n-alkane, b) mono-methyl alkane, c) di-methyl alkane, d) alkene

Current research suggests that insect hydrocarbon profiles mainly consist of n-alkanes, Z-alkenes and methyl branched alkanes [187]. It is not known where hydrocarbons are produced or in fact where they are stored [187], although production in blowfly is suspected to be under the control of ecdysteroid hormones which are in turn influenced by the juvenile hormone indirectly [194],[195]. It is the job of the abdominal cells found underneath the cuticle to produce cuticular hydrocarbons in a number of other insect species, such as locust, mosquitos and cockroaches [196]. One study showed sub-cuticular abdominal cells to be responsible for production in the case of *Drosophila*

melanogaster and it is assumed all blowfly species would be comparable [197]. Hydrocarbons are very stable once synthesised, but during production, there are numerous factors, which could result in differences. Developmental stage as well as reproductive status [192], [198]–[201] are variables known to cause this, in addition to diet and temperature [202]–[204].

Cuticular hydrocarbons seem to have differing roles in insects; ants use them to distinguish between nest mates and non-nest mates [205],[198] They are also known to be species-specific [206],[207]. The main roles seem to revolve around communication and prevention of desiccation [196],[208],[209]. The close packing of the n-alkanes allow the waterproofing of the insect and deter desiccation [209].

It is a possibility that the analysis of cuticular hydrocarbons may provide results to estimate insect age which are equally as accurate as current DNA centred research [210]–[213]. Methods are pre-existing for estimating the age of the oldest specimen and in turn estimation of PMI, hydrocarbon analysis could play an important role within this [4].

Research has previously shown encouraging results for identification of insect species. A wide variety of species have been investigated including bees [214]–[216], ants [207], [217]–[219] termites [220], blowfly [206],[221],[222], cockroaches [223],[224], wasps [225], beetles [226]–[229], mosquitos [230]–[232] and grasshoppers [233].

Hydrocarbon analysis has shown a further purpose other than taxonomic identification, it can also be used to differentiate between sexes and to age insect specimens

[191], [221], [232], [234]–[239].

1.5 Rationale and Aims

The estimation of Post Mortem Interval (PMI) is crucial within the area of forensic entomology. The various factors, which affect this calculation, are complex and can invariably encourage errors leading to the incorrect time of death being calculated. Factors such as the effect of drugs and toxins present and environmental conditions must be considered to discourage oversight of important determinants. Previous research has shown that different species have variable responses to drugs; developmental rate can be dramatically affected. Because of the number of considerations, it is very important to have accurate comparison data, as close to the conditions observed at the scene, as possible. Current research showed an increase in studies determining the effects of illegal drugs and a rising trend in the prevailing 'legal highs' or Novel Psychoactive Substances (NPS) and associated deaths, providing a focus for this research.

Emphasis will be put on the effect of NPS on the development of forensically important blowflies at different dosages and also the effect on hydrocarbon profiles.

An investigation of the effect of these drugs on oviposition and viability of any resulting eggs, determination of an appropriate medium for delivering the chemicals as well as the analysis of NPS followed by a study of larval internal samples for traces of NPS, will compliment this study.

It is hypothesized that the variables affecting larval development can also consequently mean that developmental data can be inaccurate for determining PMI. Previous research has shown encouraging results for aging developing larvae using hydrocarbon profile analysis. It is possible that the hydrocarbon profile may not be affected by drugs and toxins and therefore may prove an invaluable method for accurately aging larvae, which are affected by such chemicals.

Covered in this chapter is a thorough description of all analysis techniques and the theory behind these, alongside data interpretation methods.

2.1 Chromatography

'Chromatography' is a word used to define the separation method of compounds from a mixture. All methods of a chromatographic nature require the use of two phases, one stationary and one mobile. The stationary phase, which can be solid or liquid in nature, is immobile. The mobile phase, which can be liquid or gas and also contains the mixture in need of separation, is then passed over this and separation is triggered by the differing interactions of these compounds with the stationary phase.

The different techniques appearing under the 'Chromatography' collective term can be categorized according to the physical state of the two phases [240],[241], liquid and gas chromatography.

2.1.1 Adsorption Chromatography

Adsorption Chromatography (Figure 10 A) is also known as liquid-solid chromatography. Separation occurs when the analytes interact with the surface of the stationary phase and they are adsorbed uniquely because of the surface. This is often packed onto the inside of the column. Differing polarities of the analytes will determine each individual's attraction to the stationary phase.

These polarities lead to interactions known as Van der Waals' forces; these include hydrogen bonding, London dispersion forces and induced dipole interactions. Certain analytes can react more strongly due to these forces and therefore remain on the column for longer and consequently provide separation of the components. More polar components react more strongly with a polar stationary phase and will be adsorbed more and this is why they will remain on the column for longer period of time before being eluted [241],[242]. Most commonly used is a reverse, non-polar phase but for specialist analysis polar phases can be used. One particular stationary phase is Silica Gel, this is a polar phase because of its hydroxyl groups (-OH).

2.1.2 Partition Chromatography

In partition chromatography (Figure 10 B), the stationary phase is a liquid bonded to a solid surface, for example; a silica column in gas chromatography and the mobile phase is a gas. Mixtures will then be separated out according to their differing solubilities [243]. The solute equilibrates between the two phases; those analytes showing greater

solubility in the stationary phase will be retained for longer on the column than those favouring the mobile phase.

2.1.3 Ion-exchange Chromatography

Ion-exchange chromatography (Figure 10 C) separates components of a mixture according to the charge they hold. [241] The stationary phase is made up from anions, such as SO^{3-} or cations, such as $N(CH_3)_3^+$, which are covalently bonded, generally in the form of a resin. The mobile phase then passes through the column [243]. Analytes with an opposite charge to that of the stationary phase will be retained on the column for a longer period of time than an analyte exhibiting an equivalent charge.

2.1.4 Molecular exclusion Chromatography

Molecular exclusion chromatography (Figure 10 D) separates molecules by size. In this situation, the stationary phase is often a porous gel and ideally, there would be no attraction between the solute and stationary phase, meaning separation is performed purely based upon the physical properties of the molecule. Smaller molecules will enter the pores for a period of time and therefore remain on the column longer, larger molecules are unable to enter these pores and so move through the column quicker. This results in larger molecules such as proteins eluting from the column first, followed by smaller molecules [241].

2.1.5 Affinity Chromatography

Affinity chromatography (Figure 10 E) is when a molecule is immobilised by being covalently bonded to a stationary phase [241]. This method can be used to selectively bind molecules of interest. It is the most selective of all the chromatography mechanisms.

Separation occurs due to complexes being formed between the compounds implanted in the stationary phase (also known as a ligand) [244] and the solutes passing by which show an affinity for this compound [245]. Solutes are retained on the column until system conditions are changed in order to release the bound solutes from the complexes; this is generally achieved by a change in temperature or pH. of the environment.

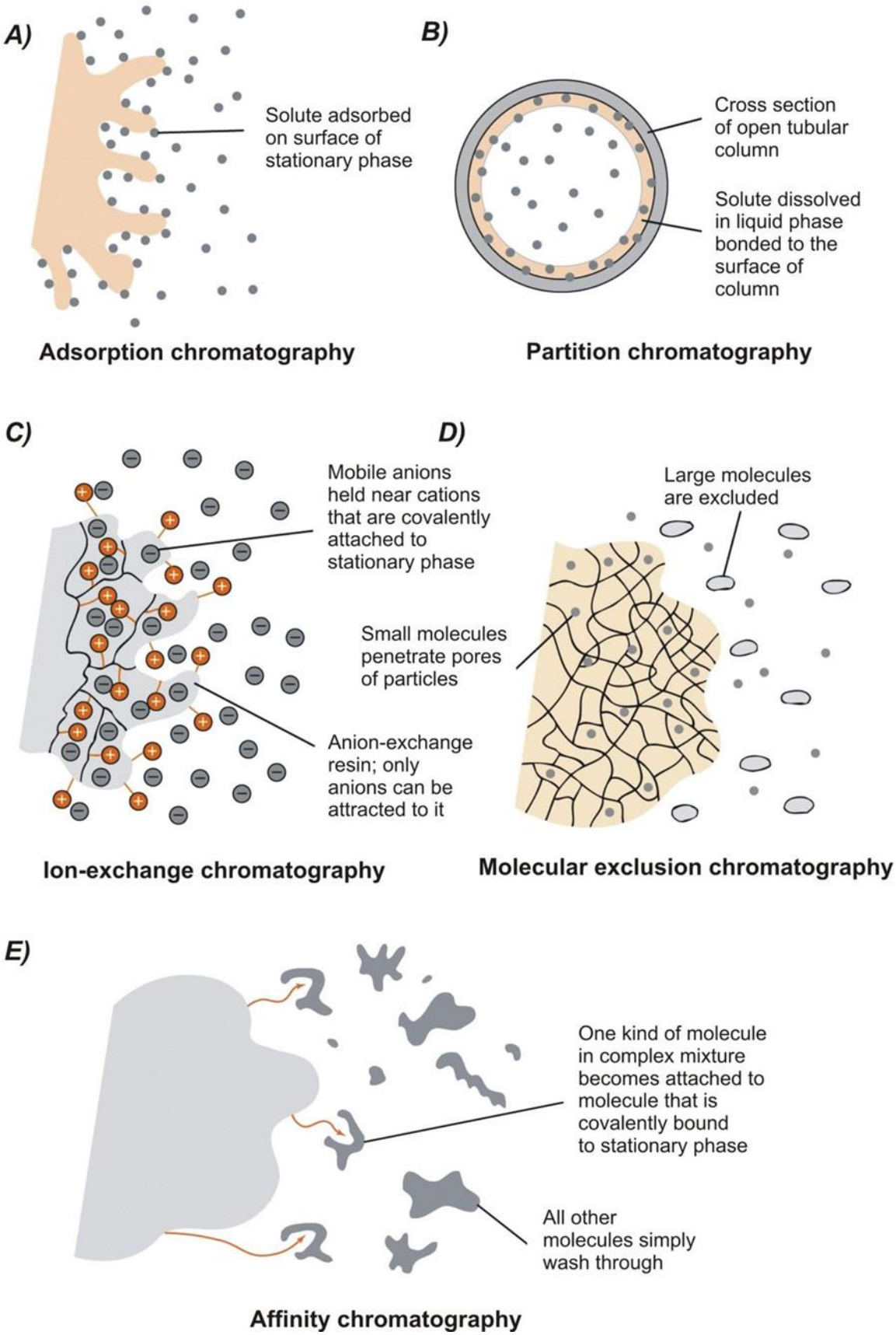


Figure 10: Mechanisms of separation, adapted without permission [241].

2.2 Gas Chromatography

Chromatographic techniques are separated into two distinct types by determining the mobile phase. This will either be liquid or gas.

Gas Chromatography was the main technique used in the research outlined in the following chapters. Irrespective of this, the fundamental stages of chromatography remain the same, as shown in Figure 11.

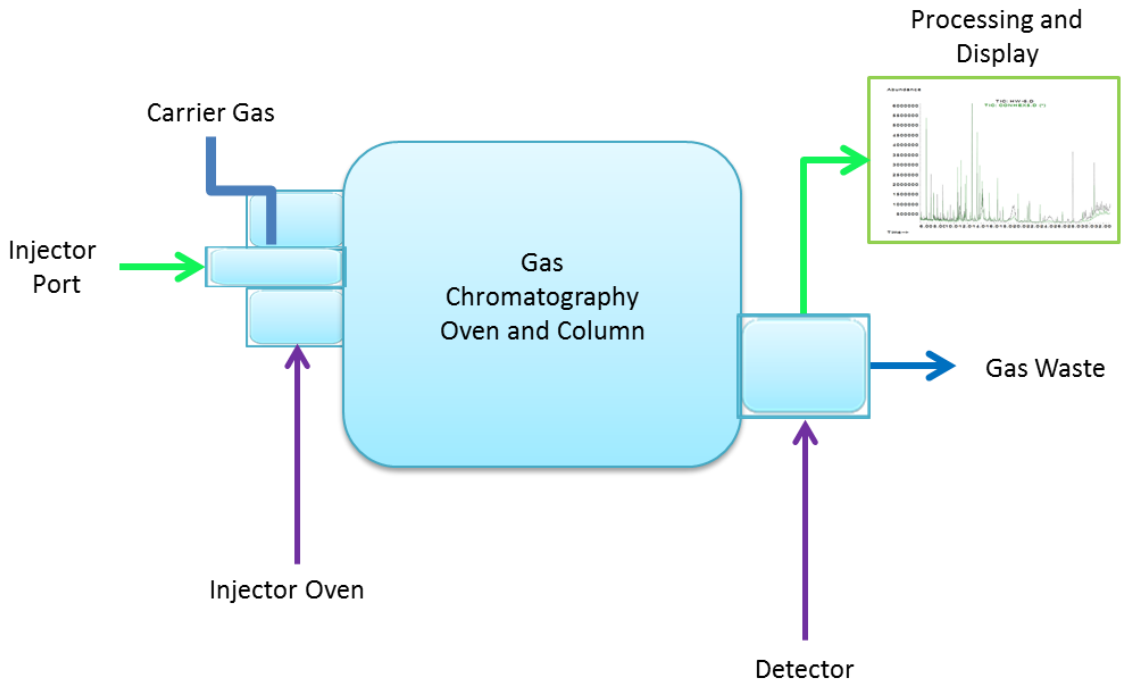


Figure 11: Schematic Gas Chromatography diagram.

Any chromatographic system must have a mobile phase into which the sample for analysis would be injected. Regarding gas chromatography, the mobile phase is gas; the sample is injected via a heated inlet, which will vaporise it. The compound is then carried through a column by the carrier gas where it is separated according to each compounds

attraction to the stationary phase of the column. The greater the interaction the solute has with the stationary phase, the longer it will take to pass through the column. The more volatile the compound is the quicker it will pass through the column. The oven temperature will vary, often starting low and ramping; this will encourage good separation and shorter run times [187]. A detector is coupled to the chromatograph to detect the elution [240]. The detector converts the compounds into peaks on a data system. The compound can be quantified in this way by calculating the area under the peak. The time the compound takes to elute (retention time) is often characteristic of the compound as it depends on the individual boiling points.

2.2.1 Split/split less injector

A sample for analysis is added to a GC system by vaporising it to form a gas. GC injections are ordinarily either split or splitless (Figure 12).

When deciding on injection type, the concentration of the sample is the main consideration. Highly concentrated samples would require a split injection whereas 'trace' or less concentrated samples would use a split less injection.

Using split mode, only some of the analytes, which have been vaporised, would end up on the column, the rest are removed in the carrier gas via a split vent outlet in the vaporisation chamber. This means a much lower amount of the initial sample ends up on the column. This method is often used to prevent overloading of the column. The ratio of analytes passed onto the column compared to analytes vented is between 1:20 and 1:500 [246].

Split less mode does not vent any of the sample, so it will be passed almost in its entirety onto the column for analysis.

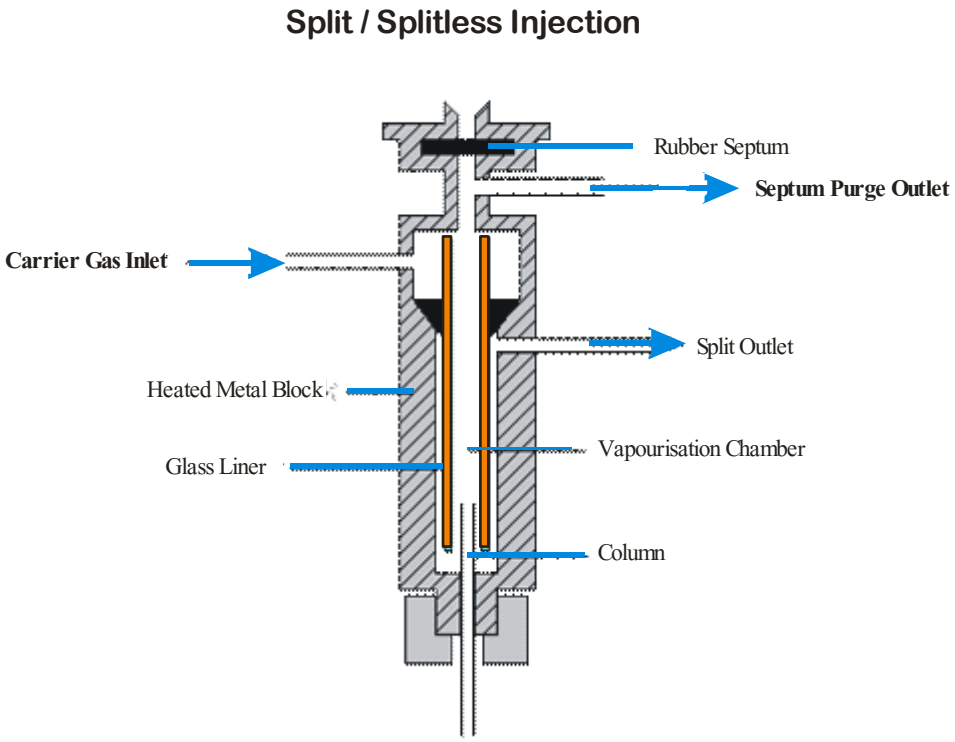


Figure 12: Split/Splitless Injection, adapted from [247].

2.2.2 Column

The sample for analysis is carried by the mobile phase through the column. This carrier gas will not interact at all with the stationary phase. The column which samples are carried through will be one of two types, packed or capillary. A packed column has the stationary phase packed inside of a glass, stainless steel or copper tube in the form of finely divided, inert, solid support material, which is then coated with a liquid stationary phase; they have a large sample capacity.

Capillary columns are open tube columns with a smaller diameter than the packed columns (Figure 13). The stationary phase is instead coated directly onto the inside of the tube. This is much more efficient as better separation can be achieved at lower temperatures and in a shorter amount of time. Cross diffusion of molecules within a sample is minimized by the decreased column diameter [248].

A capillary column was used during this research. They are often made using inert and high purity silica [249] which is then coated using polyimide to make them flexible. This is necessary for coiling within the column oven. This also means that long columns of up to 100 meters can be installed for separation. Columns coated using polyimide can only withstand temperatures up to 390 ˚C; this therefore limits the analysis of longer hydrocarbon chains.

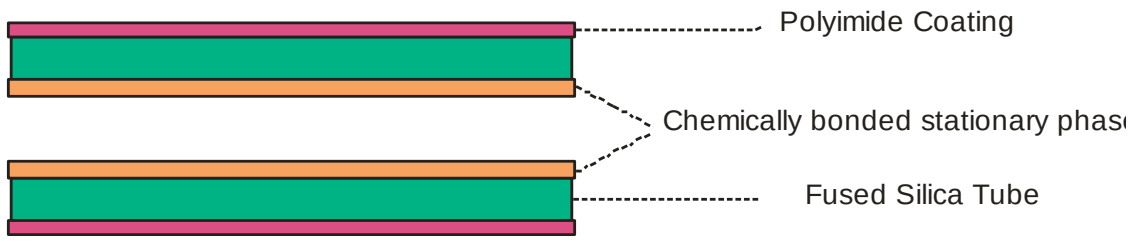


Figure 13: Cross section of a fused silica open tubular column.

Gas Chromatography is a form of partition chromatography; the stationary phase polarity will determine the degree of separation of the analytes. The stationary phase will have solid support with functional groups attached [240]. The particular functional groups attached will determine the polarity of the stationary phase. Correct selection will achieve the best possible separation. Polar columns are used to attract hydrogen bonded

compounds or those with functional groups [240]. Non-polar dimethylpolysiloxane columns are common due to their high thermal stability and low column bleed; meaning a better signal to noise ratio. Column bleed is the normal elution of the stationary phase as the column is temperature programmed [250]. Separation occurs due to the component boiling points with the more volatile components eluting first. Components are therefore eluted according to boiling point and also the number of carbons present (in increasing order) [240].

2.2.3 Column Temperature

During analysis of a component, the column temperature is important as it influences separation. There are two modes associated with column temperature.

Isothermal mode is where the column temperature remains the same throughout the analysis. This can cause problems, if the temperature is too high then co-elution can occur; resolution will be poor, but the run time would be quick. If the temperature is too low, then it will take a long time for components to elute but the separation will be adequate. Resolution however, would be poor and peaks would be broad. Some components with higher boiling points may never elute [241],[251]. Either way will result in a compromising analysis.

The second mode is known as linear temperature programming. This is where the temperature of the column oven is changed over the period of the analysis to enable efficient but good separation [241]. Method development is carried out to determine the best temperature program. This often requires a low starting temperature, which is ramped per minute to a maximum temperature. The higher the temperature the faster

the components will elute from the column and the closer together they will be on the chromatogram.

At a time when all components of interest are known to have eluted, a high temperature can be used to clean a column of remnants and any contamination ready for the next sample analysis.

2.2.4 Detection and Chromatogram

After eluting from the column, the next stage is the detection where the concentration of the sample is measured, and an electric signal response is produced. A chromatogram is then created. This plots the signal received from the detector against time, as shown in Figure 14.

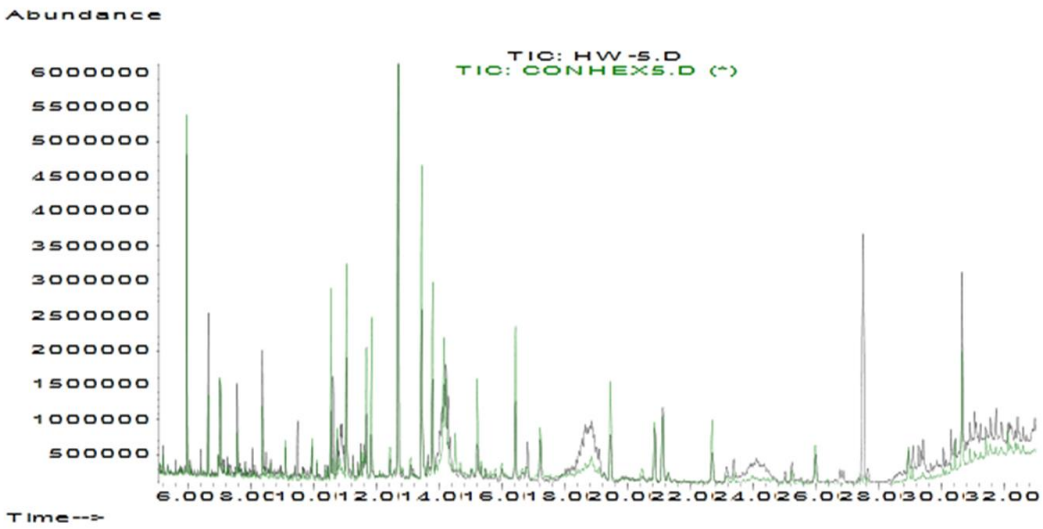


Figure 14: Example of a Chromatogram.

The position of the peaks on the chromatogram depends on each components retention time. Retention time is the amount of time the compound has spent on the column after injection. Each compound within the sample should have a different retention time

depending on its chemical structure [252]. Retention time is shown on the X-axis and the amount of the particular separated analytes on the Y-axis. Chromatograms are able to provide quantitative data for the separated mixture.

Kovats index is used when identifying hydrocarbons using GC, It enables the normalisation of retention times so the data is system independent [253]. Retention times of one compound can differ instrument to instrument. This is because the retention time is determined by a large number of variables; Firstly the identity of the analyte, the type of stationary phase used, the column length (this may not always stay the same as the column can be trimmed to remove blockages or build up), the column diameter, the flow rate and the oven temperature. Because of all these, a method is required in order to draw comparison between different GC analyses. Kovats index works by comparing the analyte retention time with standard linear alkanes [253]. The equation for this is shown below. $(I_T)_Y$ indicates the retention index with stationary phase Y at the temperature T. 'z' is the carbon number of the lower standard n-alkane. 'z+1' is the carbon number of the next homologous n-alkane and t_s is indicative of the total retention time [254],[255].

$$(I_T)_Y = 100Z + 100\frac{(\log(t_s)_x - (\log(t_s)_z}{(\log(t_s)_{z+1} - (\log(t_s)_z}$$

Equation 1: Kovats index equation.

2.3 Mass Spectroscopy

2.3.1 Overview

The mass spectrometer technique (Figure 15) uses an ion source to ionise the eluted compounds, which then fragment, and these can then be separated by deflection accorded to their mass-charge ratio (m/z). The relative abundance of these ions is detected in the mass analyser and subsequently detected then plotted against the function of m/z data in a mass spectrum; this is a plot of the mass of the fragments and their intensity which is often given as a percentage of the most abundant peak found [187]. Mass spectrometry has good sensitivity and enables determination of structural composition.

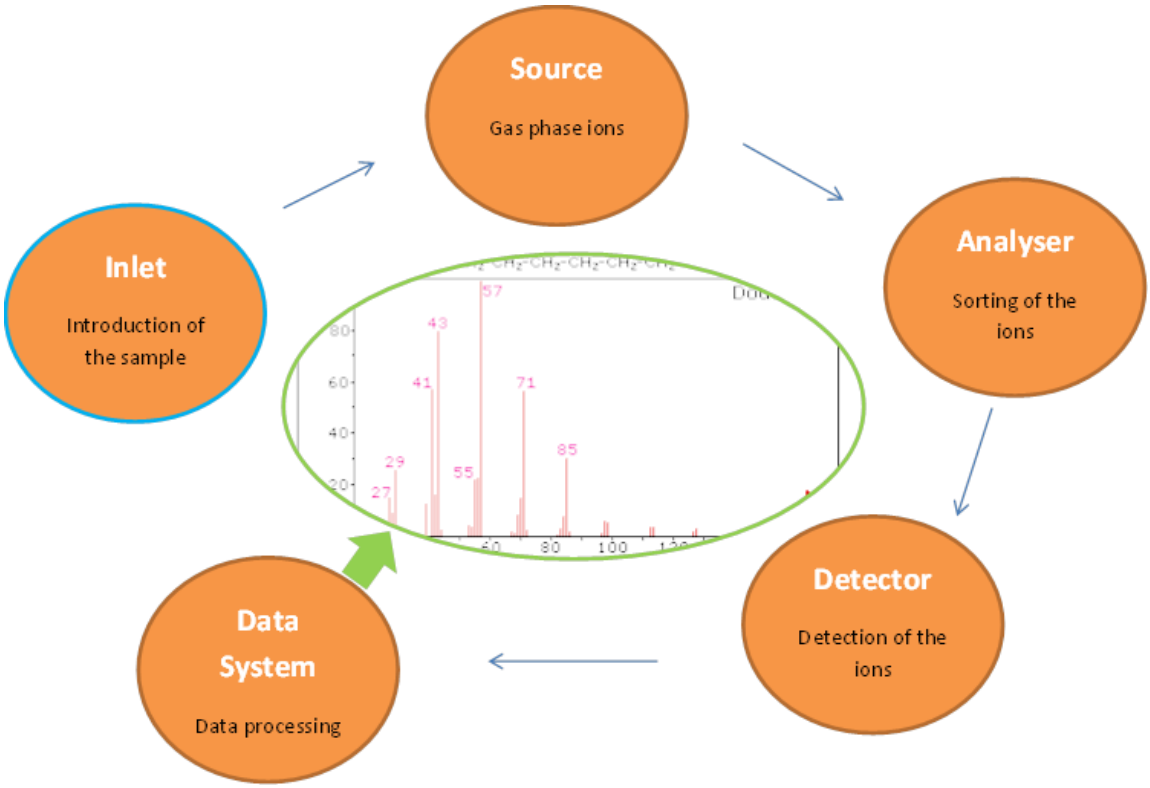


Figure 15: Steps involved in Mass Spectrometry.

2.3.2 Instrumentation

Mass spectrometry has three core stages as shown in Figure 16:

1- Ionisation

2- Analysis

3- Detection

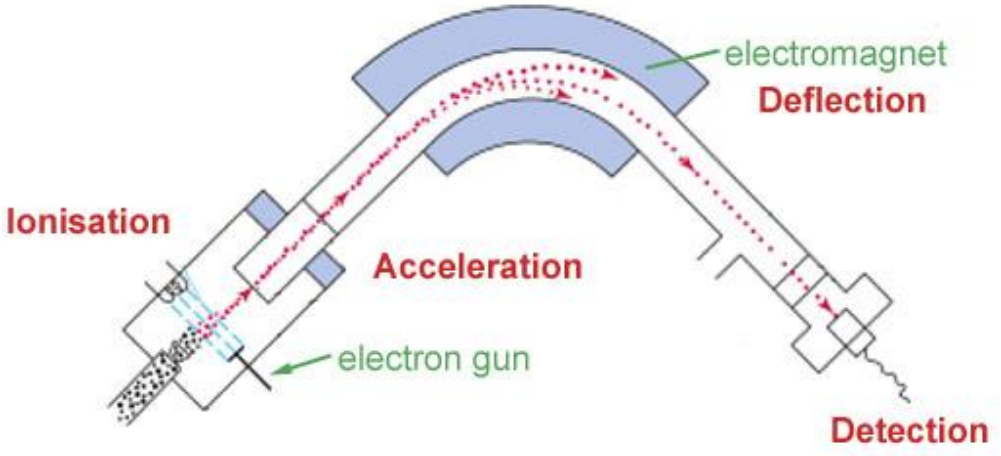


Figure 16 : The workings of Mass Spectrometry, taken from [256] without permission.

2.3.3 Ion Source

The choice of ionization method depends on the nature of the sample and the type of information required from the analysis. So-called 'soft ionization' methods such as field desorption and electrospray ionization tends to produce mass spectra with little or no fragment-ion content.

The most commonly used ion source is electron ionisation (EI) as shown below in the Figure 17, is used for non-polar compounds like hydrocarbons. Electron ionisation is

known as a ‘hard ionisation’ method. Electrons will be generated using an electrically heated metal coil or filament. The electrons make contact with the compounds causing ionisation due to loss of an electron, as shown in Equation 2.

Equation 2: Basic equation for Electronic Ionisation.

These high-energy electron collisions cause a large amount of fragmentation. The pump is used to remove neutral molecules from the MS and cations are then detected. Many fragment ions will be detected for each compound that is analysed in this manner [257]. The fragmentation of the compound is beneficial for the identification. The molecular ion will sometimes be present; this will be the ion with the highest mass/charge ratio, although it is often highly fragmented after ionisation. The molecular ion is formed during ionisation when an electron is removed during electron bombardment producing the positive ion. The ions will be repelled as shown in Figure 17; these are then focussed and continue on, to the mass analyser.

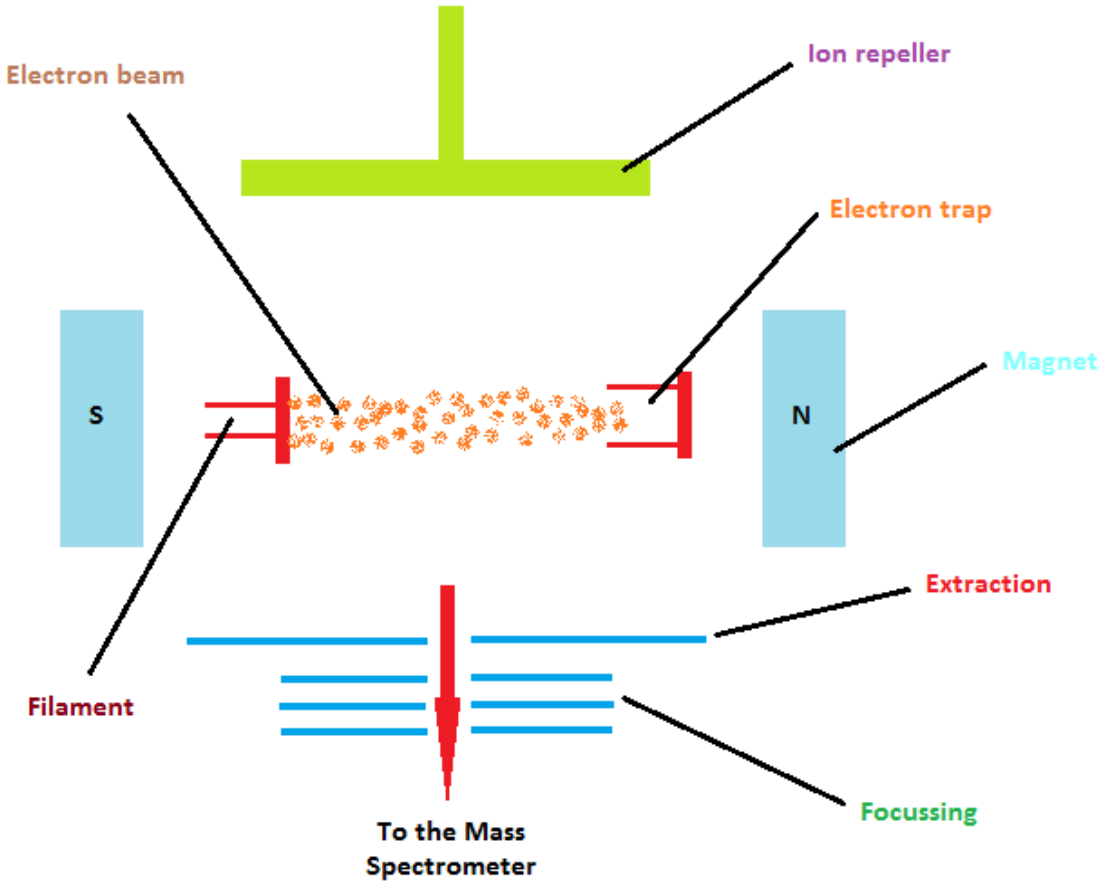


Figure 17 : Mechanism of Electronic ionisation, adapted and redrawn from [257].

2.3.4 Mass analyser

In the mass analyser, the ions are separated by their mass to charge ratio. There are a number of separation methods, the main types used with mass spectrometry are quadrupole and Time-of-flight [258],[259].

2.3.4.1 Quadrupole

Four circular rods make up a Quadrupole, which separates ions using electric fields (Figure 18). Opposite pairs are connected electronically to each other [260]. The fluctuating electronic field changes the path of some ions selecting and separating them by their mass/charge ratio, some make it through to the detector and others are deflected out by the voltage alteration and are not detected. This allows only ions with a specific mass to pass through and be detected; others are ejected as they try to pass through. When analysing using a full scan method, all ions are to be observed but in SIM (selected ion monitoring) mode then only specific ions will be detected [258],[260].

It is also possible to combine a number of quadrupoles, for example, within a triple quadrupole analyser. The first quadrupole will remove all ions except for the ion being targeted; the job of the second quadrupole is to fragment the ion further. The third quadrupole will be setup to detect the known daughter ions of the ion of interest.

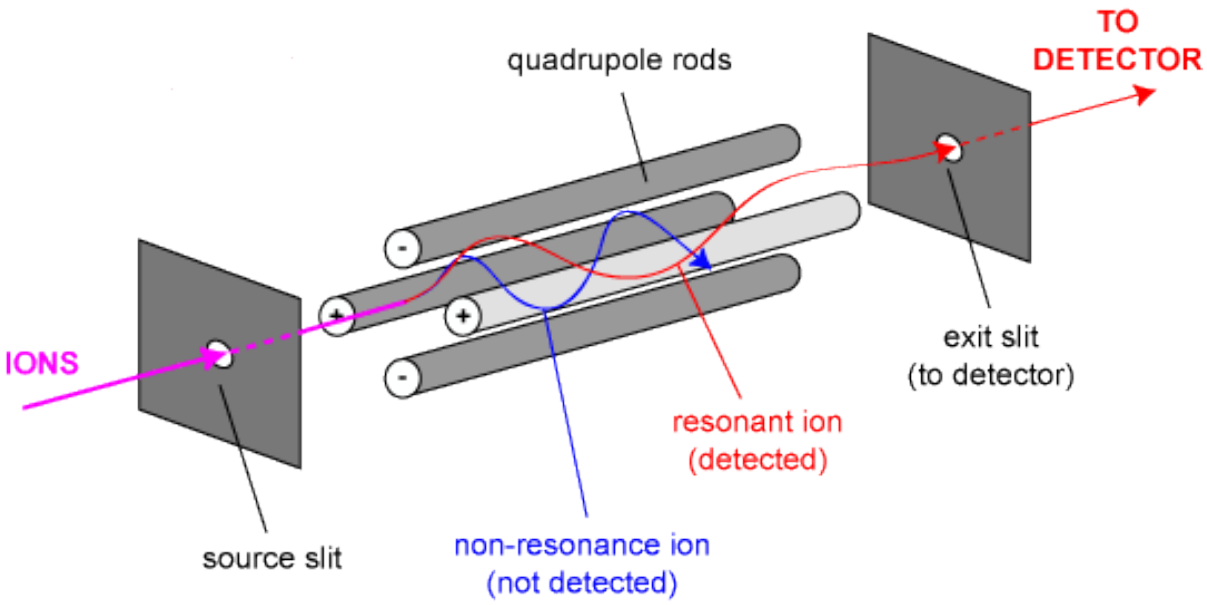


Figure 18 : Function of the Quadrupole, taken from [261] without permission.

2.3.5 Ion detector

As the ions reach the detector, the abundance of each is measured and this creates the mass spectrum, the mass is determined by the ion and the peak intensity by its abundance [259].

2.4 GC-MS Gas Chromatography- Mass Spectrometry

This is the combination of two techniques to form a singular method to analyse a mixture of compounds, including hydrocarbons or drugs qualitatively or quantitatively. The GC will separate the components and the MS will characterise each individually. GC-MS has been used since the 1960s [187] and has become the tool of choice, it provides powerful separation and identification and is also user friendly [262].

The sample is injected into the system via the injector port ('3' in Figure 19); the mobile phase carries the sample along the column where interactions will happen. Any compounds, which are non-polar, will interact with the column strongly. Those with a lower boiling point will elute from the column before those with higher boiling points, causing separation. Higher temperatures during a program ensure removal of all compounds from the column. These separated analytes then move on to the MS. Here the normal MS steps are followed, analytes are ionised to create fragments giving structural information then analysed using the quadrupole by looking at their mass to charge ratio. This information is then sent to the detector where a GC chromatogram

shows the separation of the different analytes and then each peak will have its own mass spectrum to enable identification.

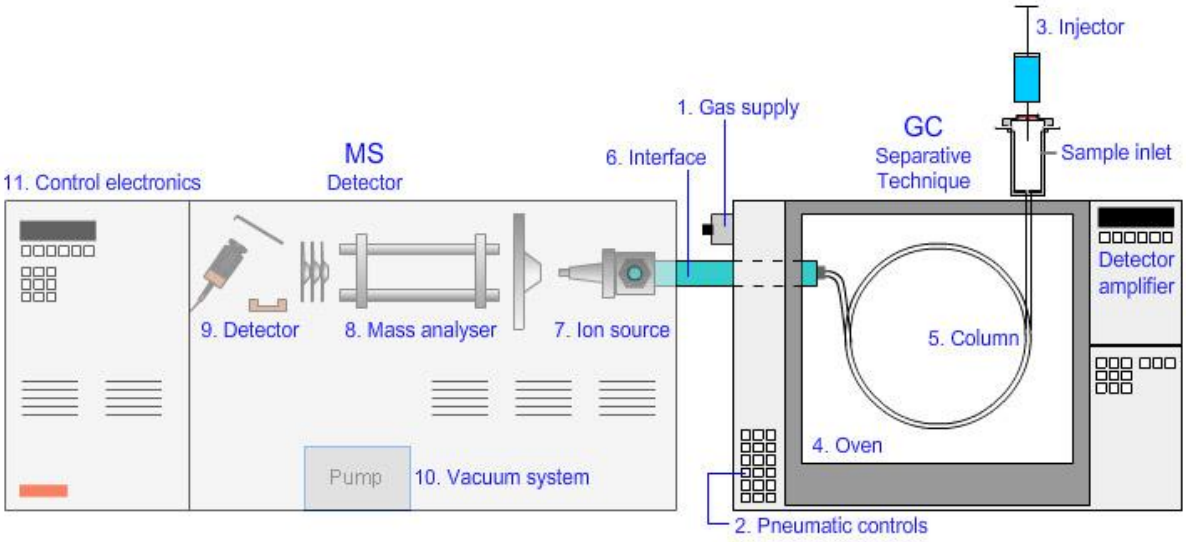


Figure 19 : Workings of GC-MS, taken without permission [263].

2.5 Liquid Chromatography – Mass Spectrometry

LC-MS is a technique that combines liquid chromatography and mass spectrometry. LC-MS can be used in the place of GC-MS to analyse a compound when the compound is non-volatile as the GC-MS cannot. This is due to the high boiling point or polarity of the compound. It also works in a similar way to GC-MS, but with a different method of ionisation. It is quite often used in MS-MS analyses, and can lead to a lower limit of detection.

As GC-MS is the focus on this thesis, this will not be expanded upon further.

2.6 Identification of Hydrocarbons

For the Identification of hydrocarbons within an extract of an insects' cuticle, it is essential to separate the larger number of compounds within a single run; this is due to the complex nature of these mixtures. GC is able to cope with this [264]. MS is then able to provide the mass of the molecule and/or the fragmented ions, GC—MS is therefore the method of choice.

It is necessary to ensure the extraction of hydrocarbons is performed with correct timings, if the insect are left in the solvent for too long then internal molecules, such as fatty acids, phospholipids and other large polar molecules will contaminate the hydrocarbon extraction and produce 'dirty' samples [187]. During the extraction process, hydrocarbons and more polar compounds are separated using silica gel, Thin-Layer Chromatography (TLC) plates can also be utilised [264].

Alkanes, alkenes, monomethyl alkanes and dimethyl alkanes can be identified using characteristic ions. Alkanes and alkenes are relatively similar, the mass to charge ratio and pattern within the lower mass region alongside the intensity of the molecular ion help to determine the hydrocarbon identity.

With Alkanes, peaks would show at 43, 57 and 71 and would be high intensity; ions of decreasing intensities would then be shown for ions 85, 99 and 113 [187]. This is characteristic of all Alkanes and the molecular ion must be used to determine the molecular weight, an example of the identification of Tetracosane is reported in Figure 20.

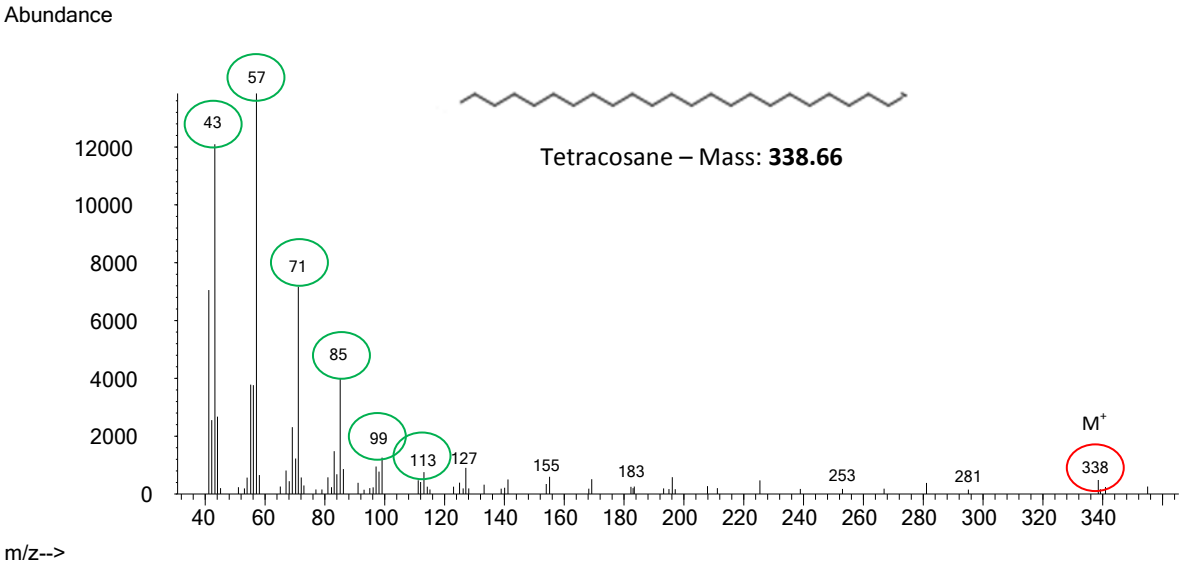


Figure 20: Mass spectrum of Tetracosane, identified with characteristic fragment ions.

The molecular ion $(M)^+$ is used to determine the chain length unless fragmentation has occurred and therefore the $(M-15)^+$ ion is observed in methylated alkanes. The reason why this ion is often observed is because it corresponds to the loss of a methyl group during fragmentation and this can then be used to calculate the molecular weight. The molecular ion is more easily recognized as it is often more intense than the fragmented ions.

Alkenes follow a similar pattern but have different distinctive high intensity ions within the lower mass region, 41, 55, 69, 83 and 97, decreasing in intensity with ions 111, 125 and 139 [187]. The molecular ion for Alkenes is more distinctive, an example of the identification of Pentacosene is reported in Figure 21.

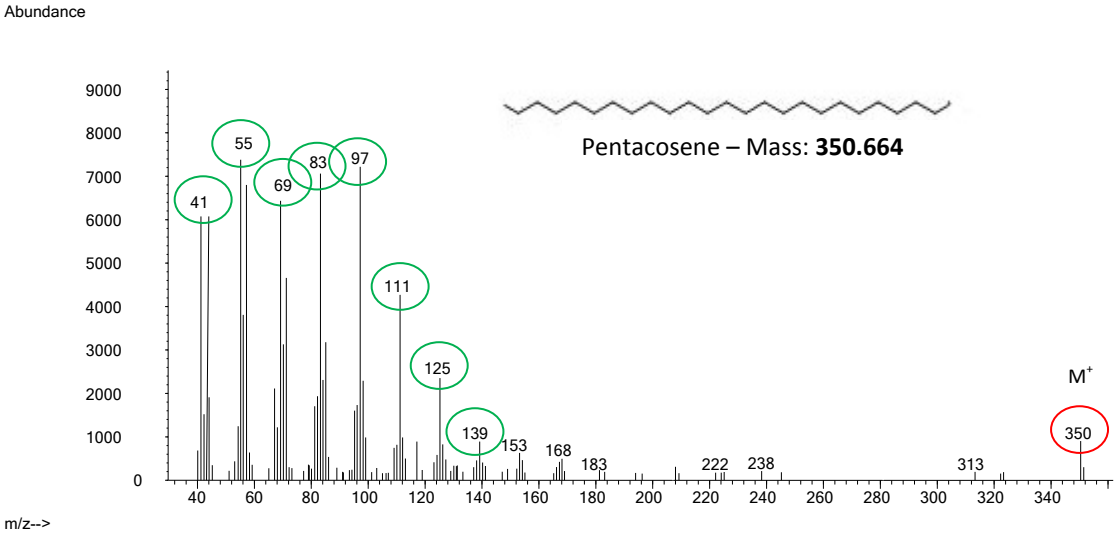


Figure 21: Mass spectrum of Pentacosene, identified with characteristic fragment ions.

Monomethyl alkanes are similar again to the hydrocarbon analysis already explained, the difference observed here is that the usual ion peaks are interrupted by a higher abundance of a pair of ions, and this signals the position of a methyl group [187]. The positioning of the methyl group determines where the ions will appear, those near the middle of the chain will be more obvious but if the methyl group were located at the beginning, the ions would peak near the M+ ion and also within the lower mass region. See Figure 22 for identification of two co-eluting mono-methyl branched alkanes. Fragmentation reveals where the chain has cleaved and is indicative of the methyl branch position, this is explained further in Figure 23 and Figure 24 where the cleavage is shown to explain fragmentation ions observed for 11MeC25 and 13MeC25.

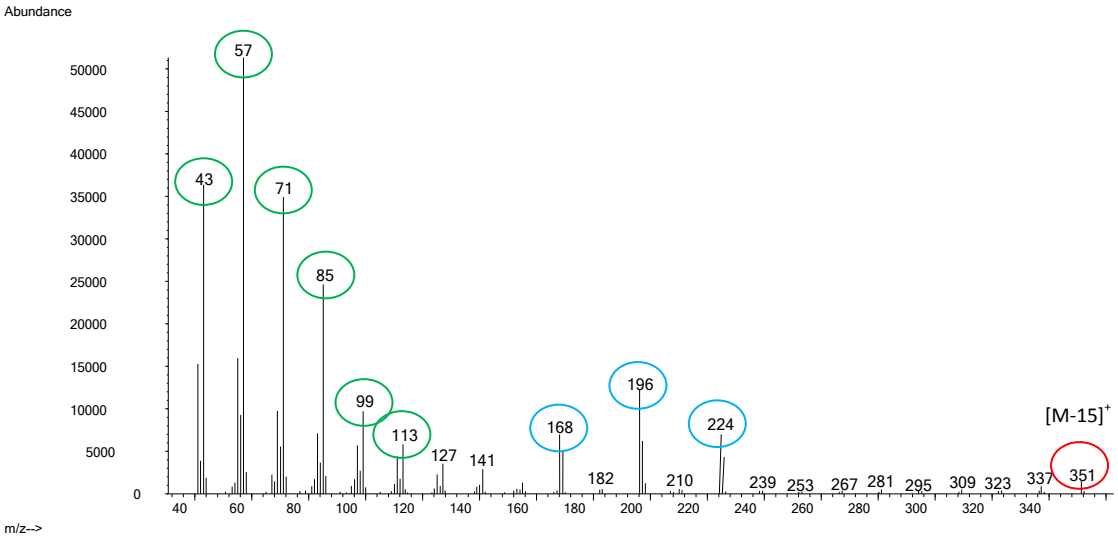


Figure 22: Mass spectrum showing the co-elution of 11MeC25 and 13MeC25, identified with characteristic fragment ions.

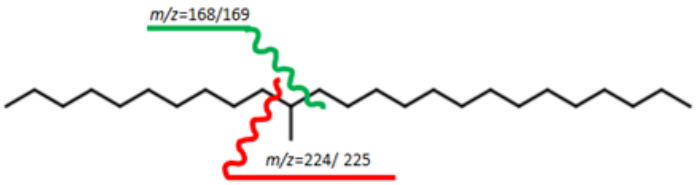


Figure 23: Chemical structure showing the cleavage producing characteristic fragmentation ions of 11MeC25.

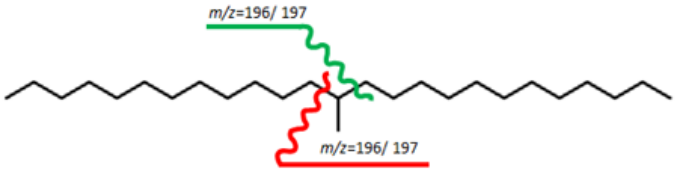


Figure 24: Chemical structure showing the cleavage producing characteristic fragmentation ions of 13MeC25.

Dimethyl alkanes will show as above but with further ion pairs interrupting the usual observed peaks [187].

Branched hydrocarbons can cause identification issues, this is because 2 isomeric compounds can elute as a single peak, and this will then complicate the MS spectra as it will show a mixture of the two [187].

2.7 Multivariate analysis

Multivariate analysis is a statistical technique, which is used to analyse data with more than one variable. There are many forms of multivariate analysis and the type used is dependent on the data produced during experimentation. Often a large amount of data is produced and this needs condensing in order to determine the result. One of the main techniques utilised throughout the duration of this research is Principle Component Analysis (PCA), this is used to group data according to any significant similarities within the data (including chromatograms) in order to reveal patterns or trends. It works by characterising a set of data via defining a small number of uncorrelated principal components. These then represent significant variability within the dataset [265].

3.1 Colony Setup and associated methods

3.1.1 Blowfly colony rearing and maintenance

A number of blowfly colonies were used over the period of this research. Initial colonies were used for preliminary studies. These species were selected due to their presence in the United Kingdom and their role as early colonizers of a cadaver.

3.1.1.1 *Lucilia sericata (Meigen 1826)*

An initial colony of *Lucilia sericata (*Figure 25*)* was kindly supplied in August of 2012 by Dr Kate Barnes, Lecturer in Forensic Biology at the University of Derby. (Location: Kedleston Road site, Derby Campus.) This colony originated from Castaway Tackle in Lincoln but was frequently supplemented with wild *L.sericata* blowfly. A second supply was also provided by Dr Kate Barnes, from the same source, in June 2014. These colonies were laboratory reared as explained further in this chapter. Milk powder, water and sugar cubes were all provided to the individual colonies and then animal tissue (usually liver, mince, pork chop or cat food) was introduced to prepare blowfly for oviposition and to provide an appropriate medium [266].

Figure 25: *Lucilia sericata*, taken from [144] without permission.

3.1.1.2 *Calliphora vomitoria (Linnaeus 1758)*

An initial colony of *Calliphora vomitoria* (Figure 26) was kindly supplied in August of 2012 by Dr Katherine Brown, Senior Lecturer at the University of Portsmouth. (Location: Ravelin House, Ravelin Park, Portsmouth, PO1 2QQ.) A second supply was provided by Dr Kate Barnes, from the University of Derby, in September 2014. These colonies were laboratory reared as explained further in this chapter. Milk powder, water and sugar cubes were all provided to the individual colonies and then animal tissue (usually liver, mince, pork chop or cat food) was introduced to prepare blowfly for oviposition and to provide an appropriate medium [266].

Figure 26: *Calliphora vomitoria*, taken from [267] without permission.

3.1.1.3 *Calliphora vicina (Robineau-Desvoidy 1863)*

A colony of *Calliphora vicina* (Figure 27) was kindly supplied in September of 2014 by Dr Kate Barnes, Lecturer in Forensic Biology at the University of Derby. (Location: Kedleston Road site, Derby Campus.) These colonies were laboratory reared as explained further within this chapter. Milk powder, water and sugar cubes were all provided to the individual colonies and then animal tissue (usually liver, mince, pork chop or cat food) was introduced to prepare blowfly for oviposition and to provide an appropriate medium [266].

Figure 27: *Calliphora vicina*, taken from [268] without permission.

Later colonies were built by catching wild flies or rearing eggs from wild flies. This enabled strong colonies to be built and supplemented as necessary. Identification was essential when building colonies in this manner and the protocol for this is outlined below. Egg collection and adult blowfly trapping took place in numerous locations around Keele University but mainly outside of Lennard-Jones Laboratories and the woodland outside Keele Hall.

3.1.2 Method for catching wild adult blowfly

This method required a handmade trap. This was produced using a plastic drinks container which was cut into two pieces and one inverted. Two further funnels were added around the mid-point and a small hole pierced into the bottom of the bottle, should rain water start to collect. Animal liver was then placed into the container in order to attract blowfly. These handmade traps were placed in areas, which were less likely to be disturbed, by both animals and humans; often these were hung in trees, (Figure 28 and Figure 29). The trap was then monitored and taken back to the laboratory for identification and sorting once a larger number of adult blowflies were inside.

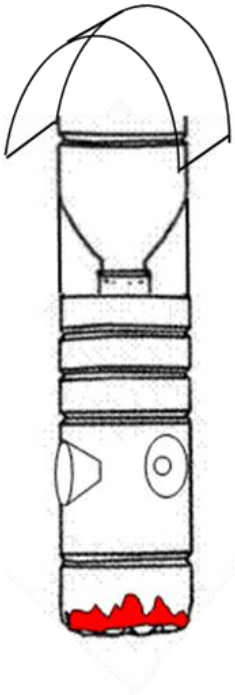


Figure 28: Diagram showing the trap setup [269].

Figure 29: The trap in-situ in two locations at Keele University.

3.1.3 Method for acquiring blowfly eggs for rearing

This method involved placing petri dishes filled with different types of food, (mainly liver, minced beef and cat food) into areas unlikely to be disturbed. These dishes were placed around Keele University in woodland areas and also around the laboratory building. The blowflies were then attracted to these and laid eggs as normal. These petri dishes were monitored daily for activity and collected once eggs were present (Figure 30).

Egg clumps were separated into individual boxes and provided with food in order for them to hatch and develop through the instars and pupate. On emergence, the now adult blowflies were identified and allocated to different rearing cages.

Figure 30: The method of egg collection in two locations at Keele University.

3.1.4 Identification

Once blowfly adults had been trapped or blowfly eggs had been reared through to adults, identification was performed before placing blowfly into colonies containing an individual species. Identification was performed using stereomicroscopy and taxonomic keys as described below.

Light microscopy is a common method used when identifying blowfly species. Taxonomic keys where morphological features are described are provided for this purpose. There are numerous published keys [270]–[274]. Once samples have been collected, they are retained for identification. Species identification requires a level of expertise and can be difficult in the case of similar species. All identification keys are shown in the appendix and are taken from a handbook provided during the forensically important Diptera identification workshop organised by Krzysztof Szpila and Andrzej Grzywacz at the Nicolaus Copernicus University, Torun, Poland. [270].

3.1.5 Rearing

Colonies were reared under normal environmental conditions in a dedicated laboratory. Temperatures were kept constant at 23°C ±1˚C and humidity heightened. It was also important to replicate a realistic summer photoperiod, 16 hours light, and 8 hours dark, so that larval development is not altered. This was delivered using a timer on the LED lights. Purpose-made cages were used for adult blowfly (Figure 31)**.** Each cage was provided with water from an adapted container, where there was no open body of water, only a material which soaked up the water which discouraged drowning. Also provided was sugar in the form of cubes and milk powder, which provides amino acids, the building blocks of protein. Pig liver with horse blood was introduced to each cage as an oviposition medium and substitute protein source. It was necessary to provide a substitute protein source to initiate vitellogenesis. This is when the fat body stimulates the release of juvenile hormones and produces Vitellogenin protein, preparing the blowfly ovaries [275], [276]. A number of different media were trialled during more unsuccessful rearing periods, this solution was the most consistently successful combination. Paper towel was used to inhibit drying out of the meat and increase the number of viable eggs.

Figure 31: Rearing cage.

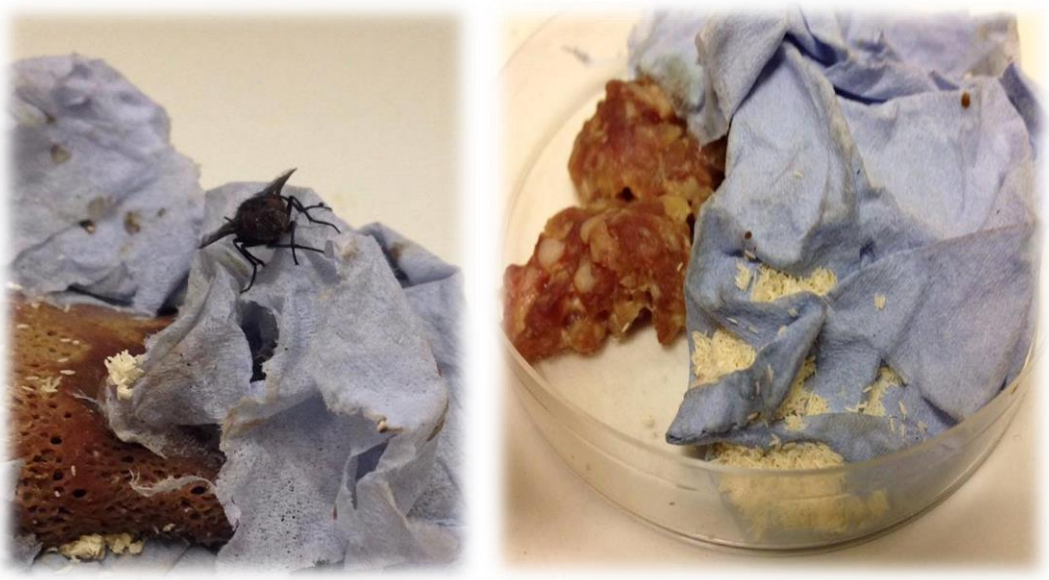


Figure 32: Egg clumps observed.

Petri dishes, now containing eggs (as shown in the photos in Figure 32) were removed from the rearing cages, normally after a two-hour period. However, this did depend on the size and strength of the colony. Eggs were then placed into purpose made boxes with air holes in the lids.

Eggs were separated into a number of boxes in order to reduce competition for food and discourage larvae moving away from the box to find alternative food sources. This encouraged larvae to not escape through the air holes during first instar. A mesh was also used in between the lid and larval mass for this purpose. Paper towel or wood shavings were placed into the bottom of the boxes, underneath the petri dishes which was then sprayed with water in order to provide a humid environment required for development.

Each box was clearly labelled with species, cage number and date the eggs were laid. These were monitored daily to ensure progression was as expected and meat was always present (Figure 33). Meat was removed when the post feeding stage was reached in order

to reduce the odorous gases formed. Meat provided during rearing was either pig's liver or minced beef.

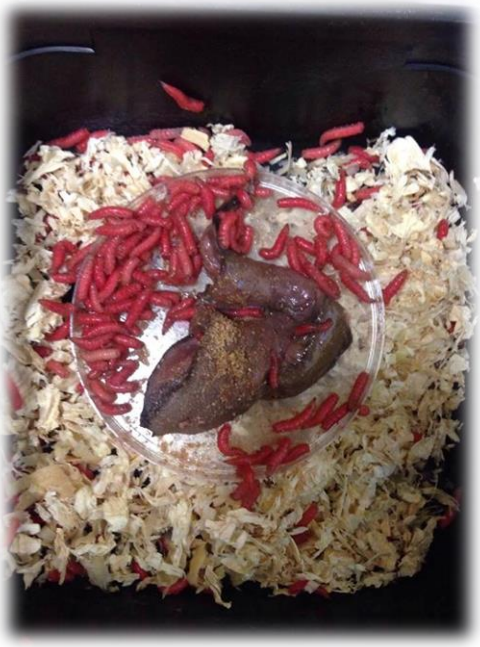


Figure 33: Larvae (coloured for visual aid) feeding from meat provided in rearing box.

A good airflow was required for the larvae, as this prevented a build-up of ammonia gas, which is produced by the larvae when they feed. A build-up of ammonia was shown during one experiment to halt the lifecycle at pupariation.

Containers of water were placed into the LMS incubator to increase humidity levels. The temperature was set at a constant 24 ±1°C.

Figure 34: Incubator setup and cups used after pupariation.

Once all larvae had pupariated, they were removed from the incubator and gently placed into plastic cups along with wood shaving or paper towel, as shown in Figure 34. Pupae require light at this stage in their lifecycle and hence were removed from the incubator. Blowfly would then start emerging and would be placed into a specific colony cage.

3.2 Materials

Shown in this section is the purchase location information for materials used during this research.

3.2.1 Selection of Novel Psychoactive substances for research

Drugs were selected for this research based upon popularity and availability; this was determined using research of many drug-user chat rooms and from asking numerous online headshops. It was also necessary to choose chemical compounds as opposed to herbal compounds.

Shown below in Figure 35 is the price of a few Novel Psychoactive substances (NPS) at the start of the research project. Those highlighted in red were chosen for research. These were popular at the time and this is reflected upon the pricing.

Figure 36 shows information on the number of 'headshops' found selling specific NPS at the time, this shows in red the NPS selected, they were readily available.

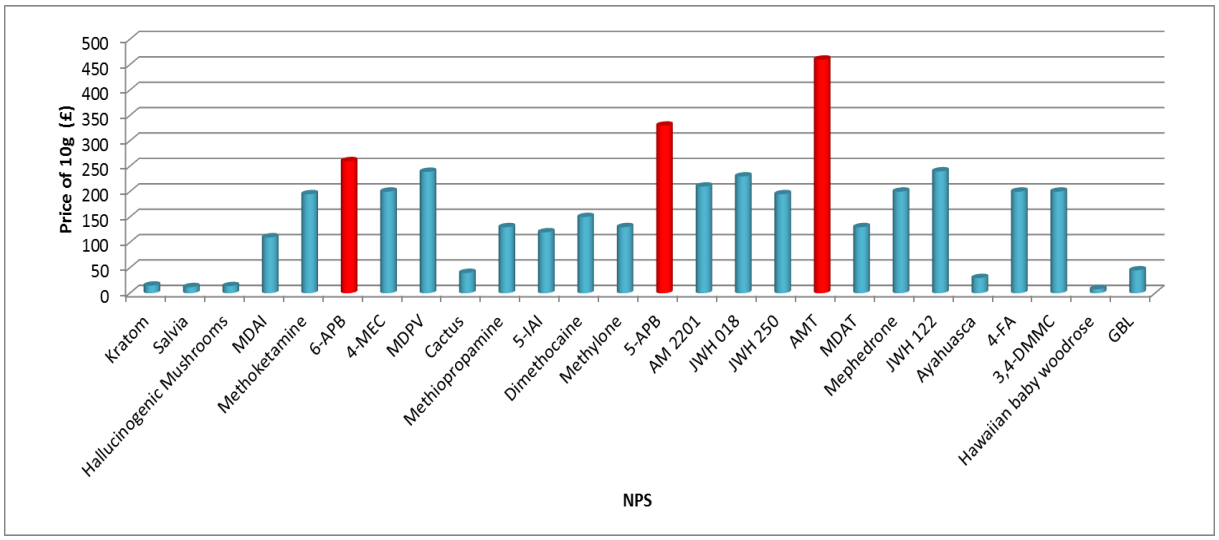


Figure 35: The prices for common NPS [172].

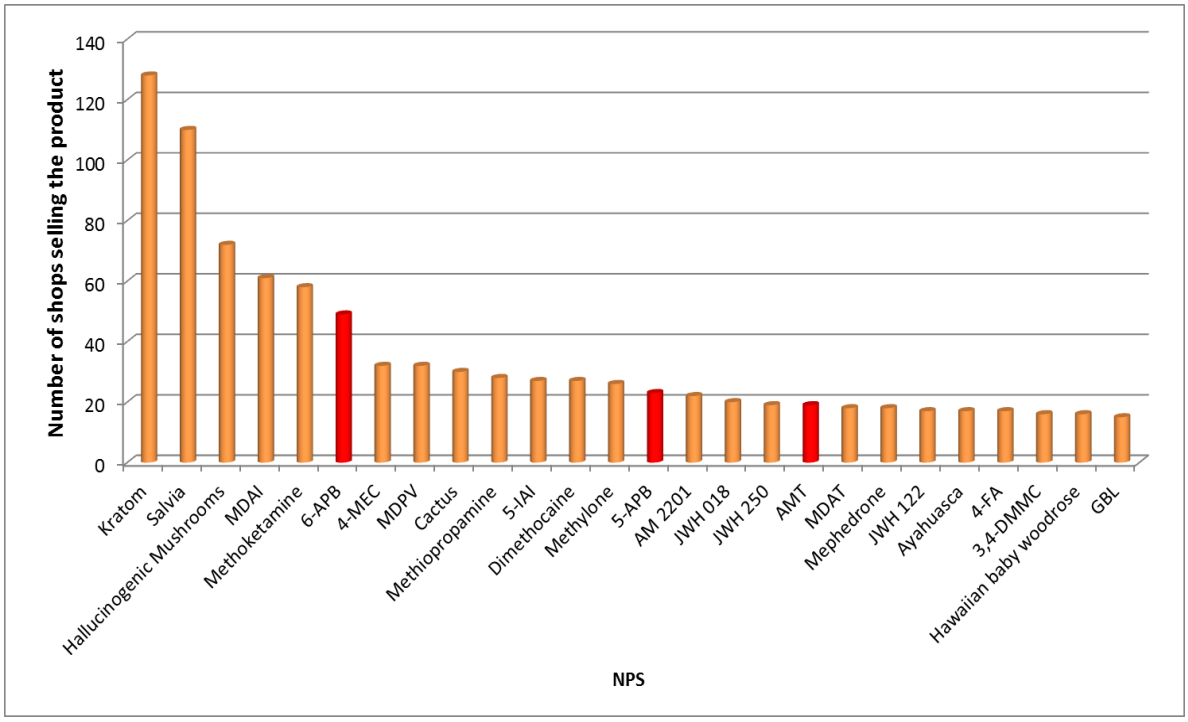


Figure 36: The number of head shops selling specific NPS [172].

3.2.2 Drug sourcing

All legal highs were purchased over the internet before the writing of legislation inhibiting use and sale. All drugs used within this research were legal at the time of purchase. Caffeine was purchased from Acros Organics, part of Thermo-Fisher at a purity of 98.5%. Paracetamol was purchased in tablet form from a local supermarket and prepared by pestle and mortar.

All other chemicals and their purchase information are shown in Table 69, found in the appendix.

All compounds were kept according to guidance of the home office and under the direction of the schedule one license kept onsite. During the period of experimentation, compounds were habitually reclassified and required specific storage and usage guidelines to be followed.

3.2.3 Solvents and chemicals

Table 4: Table showing chemicals and their purchase information.

Solvent	Use	Purchased from
n-Hexane (HPLC grade)	The extraction of hydrocarbons from the cuticle of the blowfly	Fisher Scientific (Leicestershire, United Kingdom)
Methanol (HPLC grade)	The extraction of internal components	Fisher Scientific (Leicestershire, United Kingdom)
Dichloromethane (HPLC grade)	The extraction of internal components	Fisher Scientific (Leicestershire, United Kingdom)
Ethyl acetate (HPLC grade)	The extraction of internal components	Fisher Scientific (Leicestershire, United Kingdom)
N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA)	Drug derivatisation	Sigma Aldrich (Dorset, United Kingdom)
Trifluoroacetic anhydride (TFAA)	Drug derivatisation	Sigma Aldrich (Dorset, United Kingdom)
Heptafluorobutyric acid (HFBA)	Drug derivatisation	ACROS organics
Chromatographic silica media 'Davisil'	Carrying out column chromatography	Grace Davison Discovery Sciences
Dimethyl disulphide (DMDS)	Derivatisation to determine double bonds of linear alkenes	Sigma Aldrich (Dorset, United Kingdom)
Iodine	Derivatisation to determine double bonds of linear alkenes	Sigma Aldrich (Dorset, United Kingdom)
Ammonium vanadate	Spot testing	BDH Chemicals, Poole, England
Acetone	Spot testing	BDH Chemicals, Poole, England
Sulphuric acid	Spot testing	Fisher Scientific (Leicestershire, United Kingdom)
Formaldehyde	Spot testing	Aldrich Chemicals Ltd
Selenious acid	Spot testing	BDH Chemicals
Cobalt (11) acetate tetrahydrate	Spot testing	Aldrich Chemicals Ltd
Isopropyl amine	Spot testing	ACROS organics
Ferric chloride	Spot testing	Sigma chemical company, USA
Sodium Nitroprusside	Spot testing	Analar
Sodium carbonate		Fisons scientific equipment
Glacial acetic acid	Spot testing	Sigma Aldrich (Dorset, United Kingdom)
1-bromoheptadecane	Retention-locking compound when analysing cuticular hydrocarbons	Fluka chemika
Docosane	Internal standard for hydrocarbon analysis.	Sigma chemical company

3.2.4 Cages, rearing boxes and artificial diets

The cages used for rearing were initially different laundry baskets, adapted for use as blowfly colony cages, these were sewn, and closable hand holes added. These cages were upgraded part way through the research. Cages were purchased from a Chinese company (Ningbo Sai Fu Experimental Instrument Co., Ltd.)

Boxes used for rearing were changed part way through the research project to combat issues originating from the size of the air holes. Plastic food boxes were originally used, and a square was cut into the top using a craft knife, this hole would then be covered with a mesh, either a type of netting or tights. This allowed circulation of air in and out of the rearing boxes. Larvae escaping from the boxes were a problem; new boxes were purchased from eBay to resolve this. These boxes were made primarily for using with tackle when fishing. A mesh or tights material was still used during the early instars when larvae were small enough to escape through air holes. These boxes meant that conditions within the rearing environment were kept constant across all rearing boxes for experimentation data purposes.

Nutrient agar was purchased from OXOID, Microbiology products. Defibrinated horse blood was purchased from TCS Biosciences Ltd, Buckingham UK. Sugar cubes and dried milk powder were purchased from the local supermarket.

3.2.5 Equipment

Zirconia/Silica beads (1.0mm) for breaking down the larval tissues when extracting internal compounds were purchased from Thistle Scientific. These were chosen due to having a density of 3.7g/cc (50% more dense than glass - good for spores and most tissues).

The Breville VHB014 400 W Black and Stainless Steel Hand Blender Set was used for preparing artificial agar diets. The meat was added into the large 700ml beaker and a 400-watt motor and stainless steel two-tip blade would blend it.

Incubation for rearing and samples kept at specific temperatures was carried out using the LMS incubator. This is a temperature-controlled cabinet with a range of -10°C to + 50°C.

All weight measurements were taken using the Ohaus analytical balance, model AR0640 - Adventurer. This had an accuracy of 0.0001g.

For vortexing internal samples, TopMix FB15024 by Fisher Scientific was utilised. Centrifuging was carried out using the Centrifuge 5415 D by Eppendorf. An ultrasonic bath was also used during preparation of internal samples; this was the Sonic 3000 by James products limited.

3.3 Analysis methods

3.3.1 Measurements of blowfly development

To determine the effects on blowfly development caused by the addition of substances to the food source, development must be monitored. This was performed by observing weight, length, instar and behaviour of the larvae, monitoring success of reaching adulthood, as well as concluding if the resulting adult is able to produce viable eggs. Weight and length are used to establish larval age currently, but this could be misleading if drugs are present. Experimental colonies were sampled for 10 days or until post-feeding stage of development was reached. The reasoning for this is that post feeding larvae move away from the food source and as a result of this; their weight fluctuates. Larval weight was determined by washing each larva in distilled water to ensure none of the food source remained on the sample, drying thoroughly using paper towel (Figure 37) and then weighing on an analytical balance. This was repeated ten times per sampling day and then the whole experiment repeated once more, to confirm an average, which helped to remove bias for extreme results.

Figure 37: Washing, drying and weighing of individual larvae.

Larvae are then submerged into boiling water to kill instantly (Figure 38) and enable their length to be measured with ease, using Digital Vernier Calipers (Figure 39).

Figure 38: Larvae pre and post submersion in hot water.

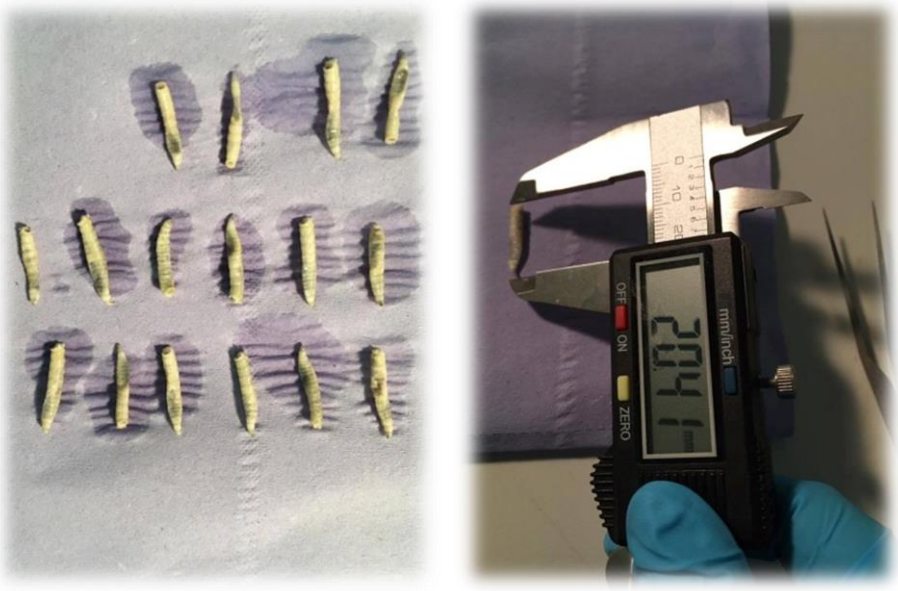


Figure 39: Larval measurement.

Larval stage was determined by looking at the number of posterior spiracle slits as shown in Figure 40 and Figure 41.

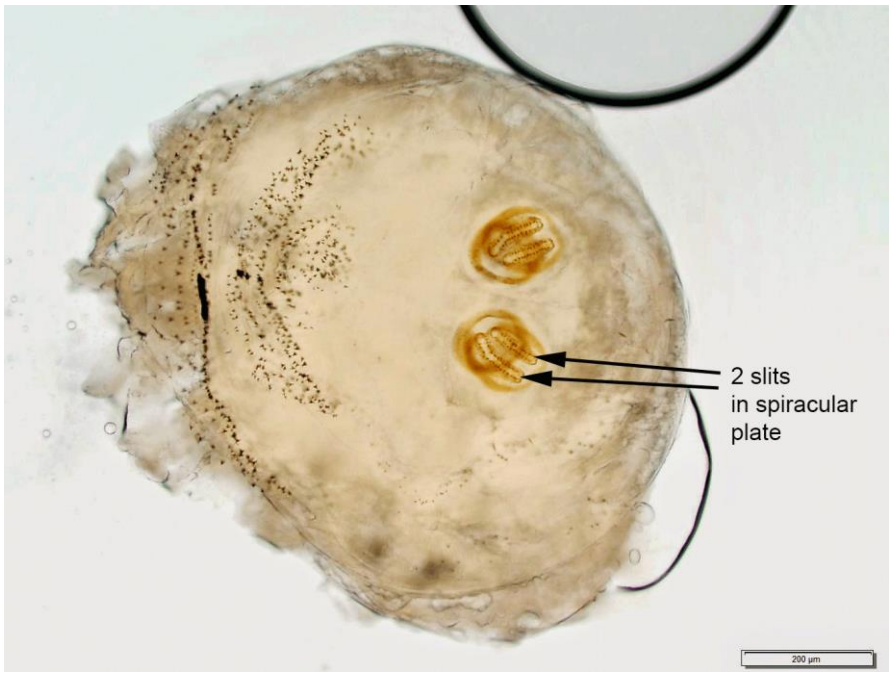


Figure 40: Microscope image of two Posterior spiracle slits.

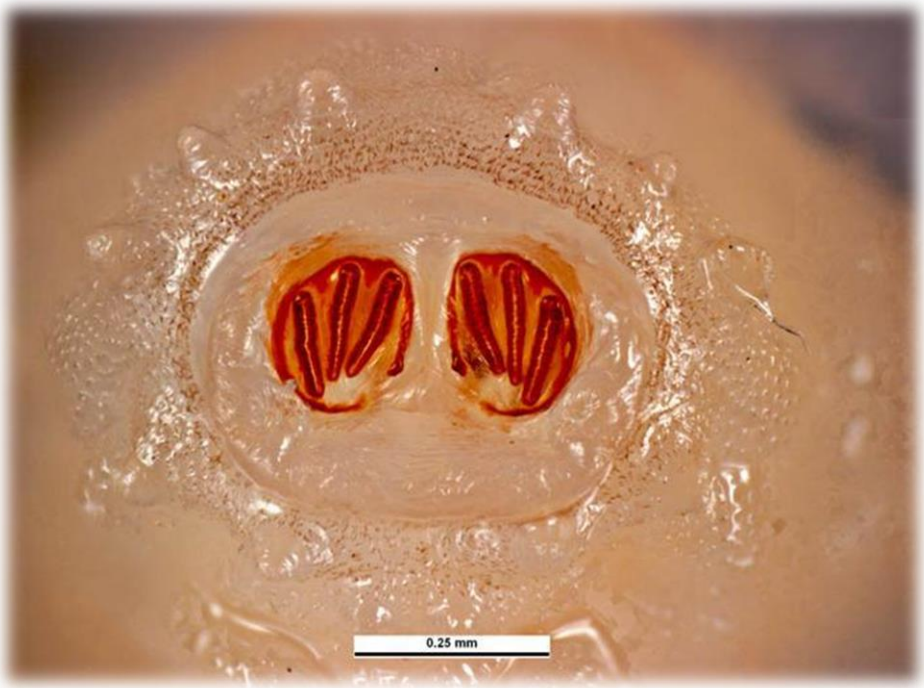


Figure 41: Microscope image of three Posterior spiracle slits.

Once the larvae reached post-feeding stage, they started to wander away from the food source in search of somewhere to pupate. This stage was often obvious due to larvae appearing at the top of the rearing box trying to escape and absence of food in the crop.

To measure the viability of future eggs from the experimental larvae, a number of emerging blowfly were kept separate for a number of days to confirm ability to lay eggs after a protein feed and the ability of those eggs to emerge.

3.3.2 Hydrocarbon extraction

Different numbers of larvae were used at different stages of the lifecycle (Table 5). This was to ensure that the concentration of hydrocarbons in the sample was high enough for detection. At first, when the larvae were very small, during the first instar, a larger

number of larvae were needed per sample. As the larva progressed through the cycle and got larger in size, smaller amounts of larvae were needed per sample. On each day, ten replicates were taken per treatment. Larvae were extracted once daily from emergence from the egg until a minimum of 50% had pupariated. This generally was a maximum of ten days at 23°C ±1˚C. In certain cases, the drugs involved in the trial caused early pupariation. The numbers of larvae corresponding to the experimental day were first killed by submerging them in hot water. Developmental data were noted and then each sample was then placed into a labelled vial and fully submerged in hexane as shown in Figure 42. This was then left for 10-15 minutes to ensure sufficient hydrocarbons were extracted into the solvent. If it was to be left for a longer period of time then internal components may start to be extracted also, this would potentially lead to contamination in the extract. To discourage contamination of the extract from components found in the food source, larvae were washed with distilled water prior to extraction. The extracts were also then run through a silica column.

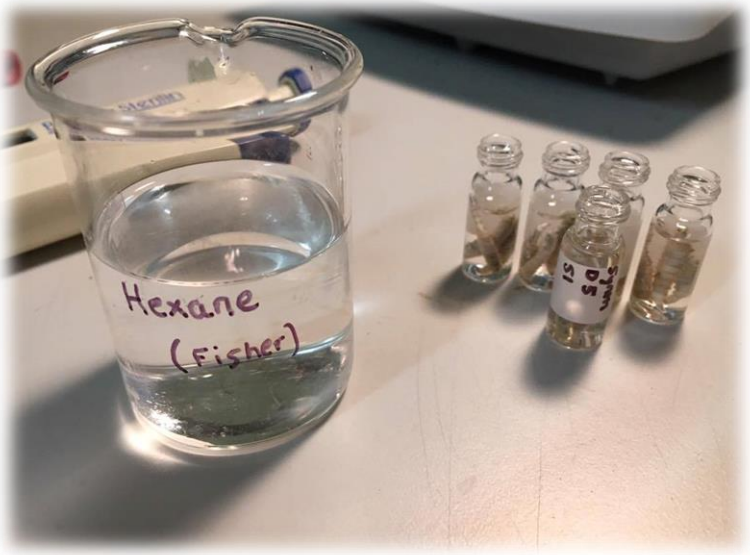


Figure 42: A selection of larval samples submerged in Hexane to extract Cuticular Hydrocarbons.

3.3.2.1 Column Chromatography

After submerging the insect as previously explained for 10-15 minutes, the resulting hexane was run through a column. Column chromatography was used in this instance to separate polar and non-polar compounds; this enabled only non-polar compounds to be injected onto the GC. This column was made using Pasteur pipettes plugged with glass wool and then a little silica gel (around 1cm), (Figure 43). Firstly, the column was wetted using 200 µl of hexane and then the extract was run through the column along with a further 500µl of hexane (Figure 44). The eluted solution was then collected and left to dry down; this increased the concentration of the hydrocarbons. 30 µl of hexane was then added and the whole extract was transferred to a glass insert inside a GC vial, which was then injected, onto the GC-MS.

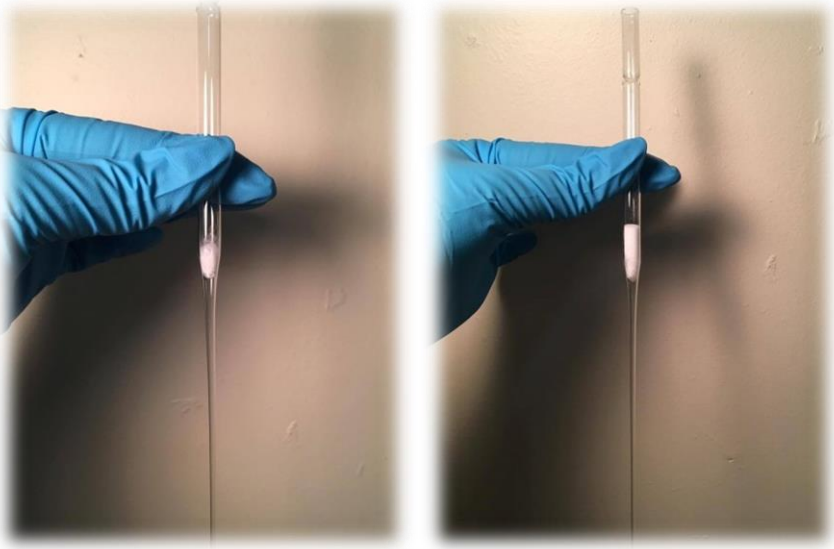


Figure 43: Preparation of the columns used in Column chromatography.

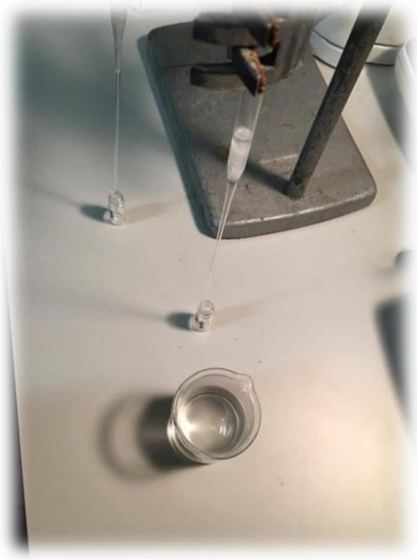


Figure 44: Column chromatography in process.

3.3.3 Overview of the daily method

On Day 0, Liver was prepped with blood and tissue paper partially covering it and placed into the fly colony cages, this was monitored, and eggs collected on tissue over a two-hour period, at this stage the Liver was removed from the cages and placed into rearing boxes and placed in the incubator.

On Day 1, the agar diet was prepared, with correct drug dosage. The fresh liver tissue was taken and blended until a meat paste was formed. 90g of this meat paste was then added to a large petri dish for each diet. In a separate beaker, 9g of agar powder and 187.5ml of distilled water were added together and mixed. This mixture was microwaved in one-minute bursts until the solution was completely clear and all agar powder had dissolved. This was then cooled to 60 °C before adding the calculated amount of drug. This mixture was then quickly added to the blended meat and mixed thoroughly with 22.5ml of blood, then left to set. All petri dishes are clearly labelled with drug and concentration. Two diets

are prepared for each population, allowing 1g of diet per larvae. At this stage, the animal tissue from the incubator was monitored closely for egg hatching. Once the majority had hatched, 600 larvae were taken using soft tweezers or a paintbrush and placed onto each of the prepared artificial drugged diets.

Between Days 1 and 10, the following procedure was carried out. At 12-hour intervals, Boxes were removed from the incubator and ten larvae selected at random from each treatment. Each larva was weighed, and the result recorded. Larvae were then returned to the population. At 24-hour intervals larvae were sampled again for both length and weight measurements. The number of larvae sampled was dependant on the sampling day (Table 5).

Table 5: Number of larvae analysed on each sampling day.

Day	**Number of larvae per sample**
1	20
2	10
3	5
4	3
5	3
6	2
7	2
8	2
9	2
10	2

Larval samples were then killed using the hot water killing method where samples are submerged in water just below boiling point for 30 seconds. The instar was noted and the length of 10 larvae was recorded. Larvae were then placed into sample vials and fully submerged in hexane. This was allowed 15 minutes before the hexane within the sampling vials was agitated and then collected. At this point, the resulting hexane was run through silica columns to remove polar compounds and contaminants, eluted hexane was collected in a labelled sample vial and left to dry down. Once completely dry, these samples were quenched and transferred to an insert for analysis on the GC-MS.

All larvae used for hydrocarbon sampling were then collected together, weighed and placed in an Eppendorf tube for analysis of internal components. This was labelled and frozen until the analysis was performed.

Once sampling was completed, some of the remaining pupae were reared through to adulthood to determine if the drug would affect them in any way (for example, ability to lay eggs).

3.3.4 Analysis of internal components

Samples were thawed, as they had previously frozen in Eppendorf tubes during sampling and then homogenised using narrow scissors. 1ml of 50:50, methanol: DCM was added to 500mg of sample, along with Zirconia/Silica beads to encourage the breakdown of larval tissues. 50mg of magnesium sulphate was also added at this stage to remove water, which was causing the solvent mixture to form two layers. The sample was then vortexed and sonicated in an ultra-sonic water bath for 15 minutes. The solvent mixture was

chosen due to experimentation with sample drug solubility. The sample was then centrifuged to encourage separation. The resulting liquid was separated for analysis, dried down and then derivatized, before being rehydrated within an insert to enable auto-sampling with the GC-MS.

3.3.5 Chemical analysis: GC-MS

All extracts were analysed using Agilent Technologies 6890N Network GC system with a split/split less injector at 250 ^{o}C, a VF-5HT capillary column (30 m x 0.25 mm ID, 0.1 µm film thickness) and coupled to an Agilent 5973 Network Mass Selective Detector. Elution was carried out with Helium at 1 mL/min. The mass spectrometer was operated in Electron Ionisation at 70 eV, scanning from 40 – 800 amu at 1.5 scans s^{-1}.

3.3.5.1. Drug analysis program

The oven temperature program for analysis of drugs and internal samples was as follows: hold at 100 ˚C for 3 minutes then ramp to 140 ˚C at 20 ˚C/min and hold for 2 minutes. Ramp again to 150 ˚C at 5 ˚C/min and hold for 2 minutes. Ramp to 160 ˚C at 5 ˚C/min and hold for 5 minutes. Finally, ramp to 250 ˚C at 20 ˚C/min. Elution was carried out at 1mL/min with helium. This program was chosen in order to separate peaks eluting closely together. Agilent Chemstation software enabled analysis of the data as shown in chapter seven. This is also displayed in Figure 45.

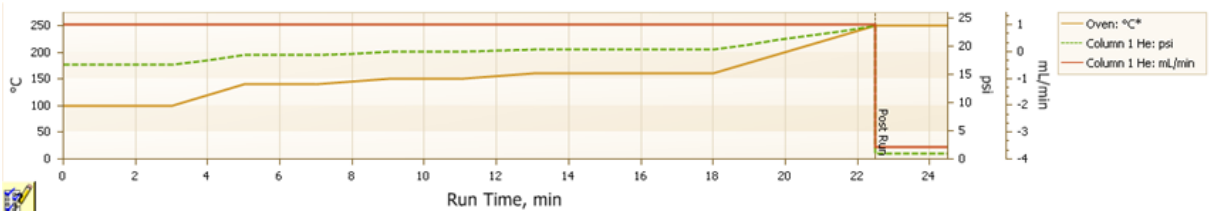


Figure 45: Drug analysis oven temperature program.

3.3.5.2 Hydrocarbon analysis program

The oven temperature for hydrocarbon samples was programmed to be held at 50 ^{o}C for 2 minutes then ramped to 200 ^{o}C at 25 ^{o}C/minute, then from 200 ^{o}C to 260 ^{o}C at 3 ^{o}C/minute and finally from 260 ^{o}C to 320 ^{o}C where it was held for 2minutes. Agilent Chemstation software was used to analyse the resulting data, identification of the alkanes, alkenes and branched alkanes using the molecular ion and the fragments ions present was possible as shown in section 2.6. This is also displayed in Figure 46.

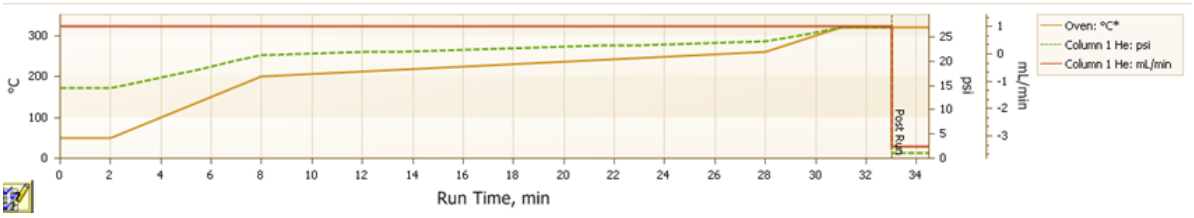


Figure 46: Hydrocarbon analysis oven temperature program.

3.3.5.3 Methylthiolation

The mass spectra of positional isomers found in hydrocarbon profiles (linear alkenes) are not easily recognised and it was therefore difficult to determine the position of the double bond during gas chromatography analysis. This is normally due to a lack of cleavage between carbon-carbon double bonds. The reaction described in previous

research utilises Dimethyl disulphide (DMDS) to enable the derivatisation of linear alkenes in an iodine catalysed addition reaction [277], [278]. This produces a mass spectrum with two abundant diagnostic ions on electron bombardment and the cleavage is shown [279] , (Figure 47). From the fragments obtained, the position of the double bond can be deduced. These derivatives are stable under the GC conditions and have a clearly visible molecular ion [278], [280]. This derivatisation reaction was carried out on one set of immature life stage samples; it was assumed that the positions of the double bonds determined would stay the same. Samples were taken, and these were extracted to obtain the hydrocarbons. To derivatize with DMDS firstly a solution of iodine in ether (5%) was added to each sample vial, followed by 100 µL of DMDS. These samples were exposed to nitrogen gas before being capped and put into an oven at 40˚C for at least 8 hours, preferably overnight.

To quench the reaction, $Na_2S_2O_3$ in aqueous form was added to the vials. This stops the reaction by removing the iodine, this forms an organic layer. This was then submitted to the GC-MS for analysis using the usual program. Temperature during the last few minutes was increased to ensure that the DMDS derivatives, which are heavier, elute from the column. The expected reaction scheme is shown in the diagram below. The dashed line shows the position where the molecule is expected to break forming two fragment ions [280].

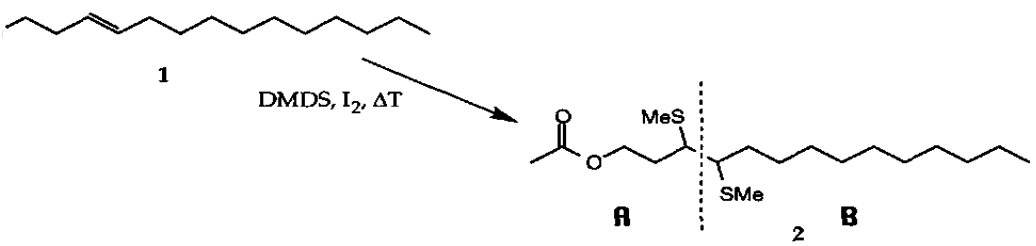


Figure 47: The suspected position of the double bond producing two fragments after the reaction with DMDS [280].

3.4 Statistical Analysis

Collected data were analysed using two statistical packages, R (version 3.4.0) and Unscrambler (version 10.5).

3.4.1 Principle Component Analysis

Principle component analysis (PCA) is an exploratory data analysis technique providing visual plots to find patterns within a dataset. It is also known as a projection method, this is because data provided to the algorithm which can have a number of variables and is projected onto a smaller number of latent variables known as principle components.

Each principle component will contain a percentage of the total information provided in the original data, the first PC contains the most and each PC after this contains less information.

The plot resulting from these PC's can provide information on the relationships between variables and groupings of samples, showing both similarities and differences can help decide the number of sources of variation within a dataset. This method allows interpretation of large and complex datasets, for example, chromatographic or spectral data. Often the first component contains useful information and later components describe mainly noise. Analysis of the initial principle components, as opposed to the dataset as a whole, helps to remove unwanted noise and ensures it is not mistaken for information.

Datasets which are input for analysis require transformations. In relation to developmental data including lengths and weights, variables were weighted in the form

of standardization. This gives all the variables the same variance and means that all the variables influence the principle components. This method of weighting is useful when dealing with variables measured in different units, different types of data or differing ranges. Considerations for spectroscopic data include the correction of the baseline; correlation optimized warping (for when peaks need aligning) and normalising. Normalizing essentially scales samples in order to get all data on approximately the same scale. The normalization method of choice when analysing the cuticular hydrocarbon profile is peak normalisation. This is where the peaks are scaled to a specified peak, in this example; the internal standard. The software used for this analysis was

3.4.2 Mixed Effects model

A mixed effects model is a statistical model, which enables the analysis of both fixed and random effects with repeated data measurements.

3.4.3 Tukey Tests

A Tukey test is a type of multiple comparison test. It compares all possible pairs and then enables identification of any difference which is greater than the expected standard error, this is shown as significant. It assumes the observations which are being tested are independent, normally distributed and have equal variance. Normality and distribution of data was tested before analysis using this method.

This chapter will address all initial experiments undertaken to allow the smooth and accurate running of the experiments described in the following chapters. Some of these experiments also provide useful and relevant information for future research.

4.1 Aims and objectives

The aim of this chapter is to establish experimental protocols to allow further experiments to determine the effect of Novel Psychoactive Substances on blowfly development, cuticular hydrocarbon profiles and to detect the presence of these substances internally.

Each section will be discussed individually due to the non-continuous nature of the chapter. Any relevant methods not already pre-defined will first be outlined, followed by results and a discussion of this and finally concluding remarks.

4.2 Mass determination for egg batches

Specimens can be counted and transferred for experimentation as eggs or larvae. When using blowfly eggs, a mass is easier and more reliable to transfer than a number. Eggs are extremely delicate and small, this makes them not only difficult to pick up and count but also susceptible to damage during this process. This can lead to eggs resulting in fewer larvae and this can alter experimental variables. For future experiments concerning eggs rather than larvae, a mass of eggs was used as opposed to a number. The aim of this experiment is to determine an accurate average egg mass for 100 eggs.

To determine the mass of the eggs, 100 eggs were counted into weighing boats and this was repeated ten times. These eggs were then weighed using the Ohaus analytical balance; model AR0640 – Adventurer and an average of these weights was used to represent 100 eggs in future experiments.

4.2.1 Results of Mass determination for egg batches

Determination of the weight of egg masses is shown in the table below. An average of these values was used as opposed to the manual counting of all blowfly eggs which may cause egg damage leading to mortality prior to reaching the larval stage. Use of an egg mass is also a more efficient method when working with blowfly egg masses.

Table 6: Calculation of an average mass for 100 individuals.

Sample Number (all 100 eggs)	Mass (g)
1	0.0438
2	0.0523
3	0.0498
4	0.0552
5	0.0541
6	0.0429
7	0.049
8	0.0513
9	0.0533
10	0.0504
Average	**0.05021**

4.2.2 Conclusion

One hundred eggs will, from this point, equate to 0.05 g; and 1000 eggs will be 0.5 g. This was utilised when the procedures started initially with eggs rather than the larval stages. Standard deviation for this samples set was calculated to be 0.0041 g.

4.3 Comparison of the effect of Hot Water Killing method on GCMS Cuticular Hydrocarbon extraction in blowflies for forensic analysis

Larval length is currently used to age species found at a crime scene. The maximum length of a larva is known to correspond to a specific instar life cycle stage under certain conditions. Larval length can be affected by many variables including killing method, protocols for sampling and storage must be followed to minimize the unknown amount of larval shrinkage or expansion that may be caused [281]. The process used to ensure the larvae do not curl and enable easiest measurement of length is known as Hot Water Killing (HWK). The larvae are dropped into water just below boiling point and then removed before 30 seconds. Following this protocol yields both reliable and reproducible data.

It would be beneficial to find out if this process used, destroys or degrades the hydrocarbon profile on the cuticle of the insect. If the hydrocarbon profile is not destroyed, then this will enable the same samples collected for larval length to then be used for hydrocarbon profile analysis. It should be noted that it is known that the larvae used for hydrocarbon profiling killed using hexane cannot be used for larval length analysis due to curling of the larvae, possibly because they do not die immediately. This would be an advantage as further collection is not necessary and where fewer numbers of larvae are present, the analysis can still take place.

Method

Ten, third instar maggots were taken and killed using the HWK method; water is boiled and then monitored to ensure the correct temperature of 80°C is achieved before the

larvae are submerged. After 30 seconds, larvae are removed, and then immersed in hexane and extracted using the usual extraction method detailed in Chapter 3. Ten additional larvae from the same batch were immersed in hexane to kill them and extracted as detailed in chapter three for cuticular hydrocarbon analysis. The results from these two analyses were then compared to see if the profiles differ.

4.3.1 Results comparing the hydrocarbon profile when using the Hot Water Killing method

Shown in Figure 48 is an example chromatogram from each method described overlaid, while Figure 49 is shown stacked to allow comparison of the hydrocarbons present, as opposed to the concentration of hydrocarbons present.

The hydrocarbons shown in the chromatogram do not appear to change with differing sample treatment. The samples killed by submerging them in hot water seem to show a slightly lower concentration of cuticular hydrocarbons but not a difference large enough to cause any difficulty during analysis.

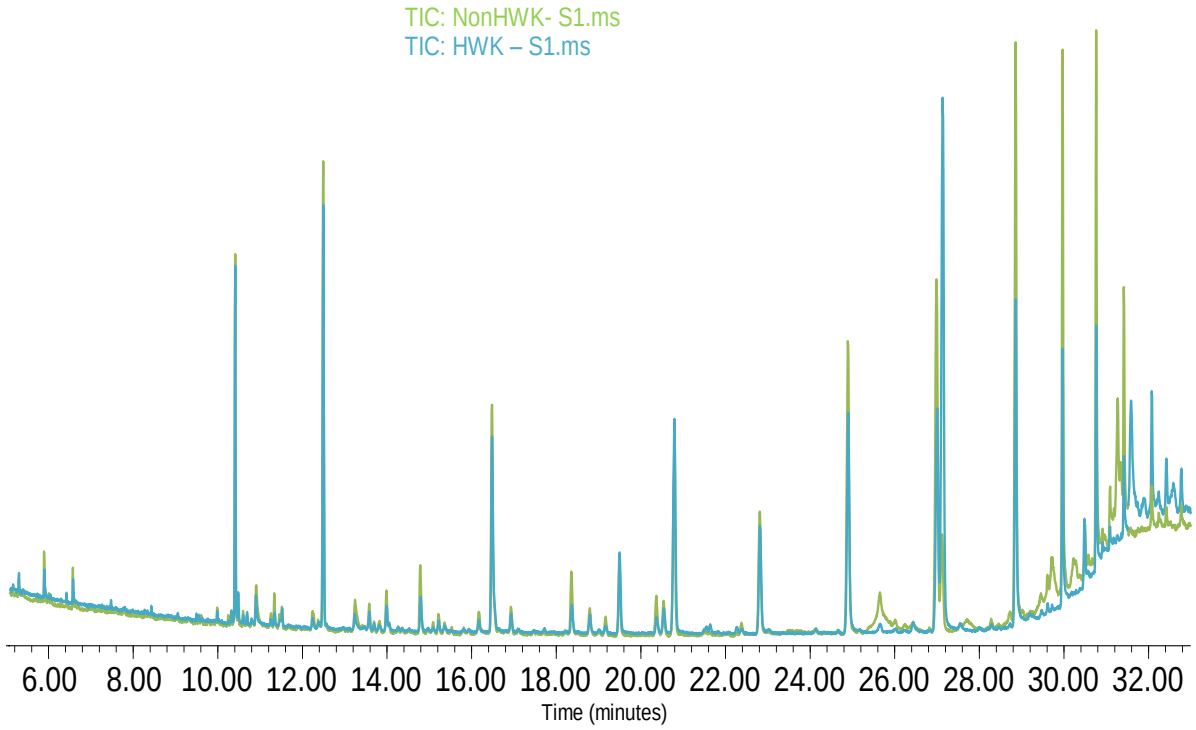


Figure 48: GC chromatogram showing the differences in Cuticular Hydrocarbons when comparing larval hot water killing method.

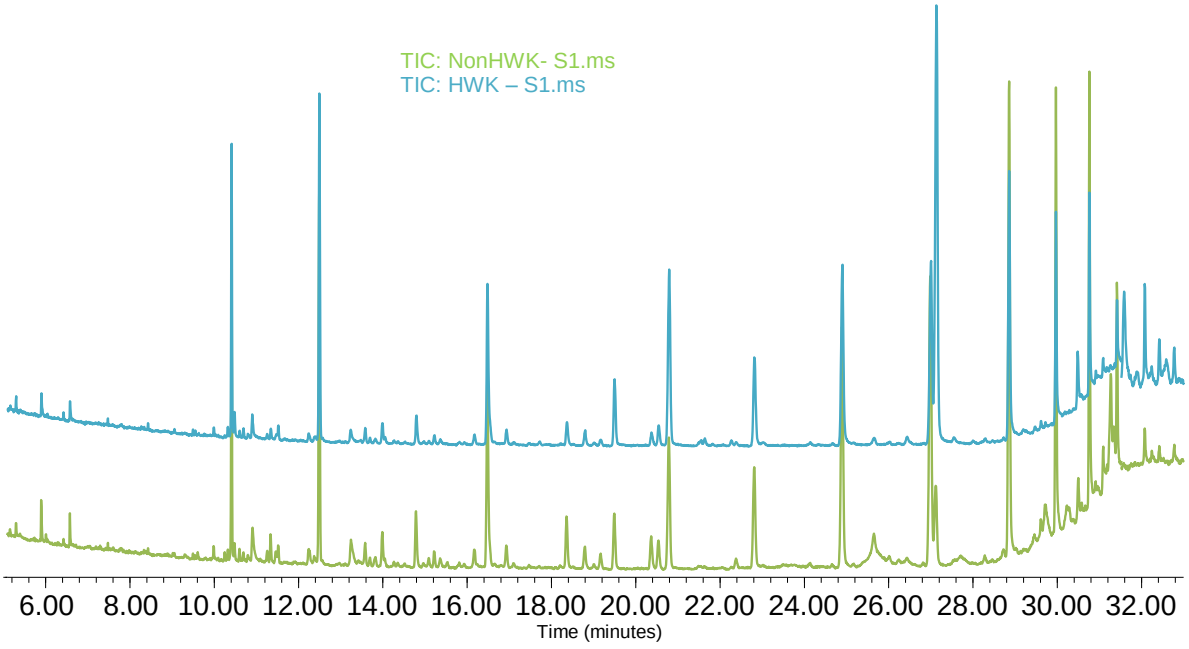


Figure 49: Stacked GC chromatogram showing the differences in Cuticular Hydrocarbons when comparing hot water larval killing method.

Chromatograms were also analysed using Principle component analysis; PC 1 and PC 2 are plotted in Figure 50 and together describe 96% of the population variance. Most samples cluster together showing similarities within the chromatograms of the 20 samples. Four samples were shown not to cluster, three of which were samples killed by submerging in hexane only and one of which is from the hot water killing method. One sample, shown circled in the PCA plot should be ignored. When the chromatogram was observed, it only showed column bleed.

To explain the clustering further, a dendrogram is shown in Figure 51. This is a hierarchical clustering method providing visualisation of the proximity of samples to each other. Clusters with a high portion of similarity to one another are coded with colours. Distances are calculated using Euclidean distance, which takes into account the difference between two samples based on the magnitude of changes within the sample levels. This shows how different Sample 6 (the previously circled sample) is from the others analysed. Sample 1, 5 and hot water killed sample 6 are different again but more alike each other, not so similar however, that they are coded using the same colour.

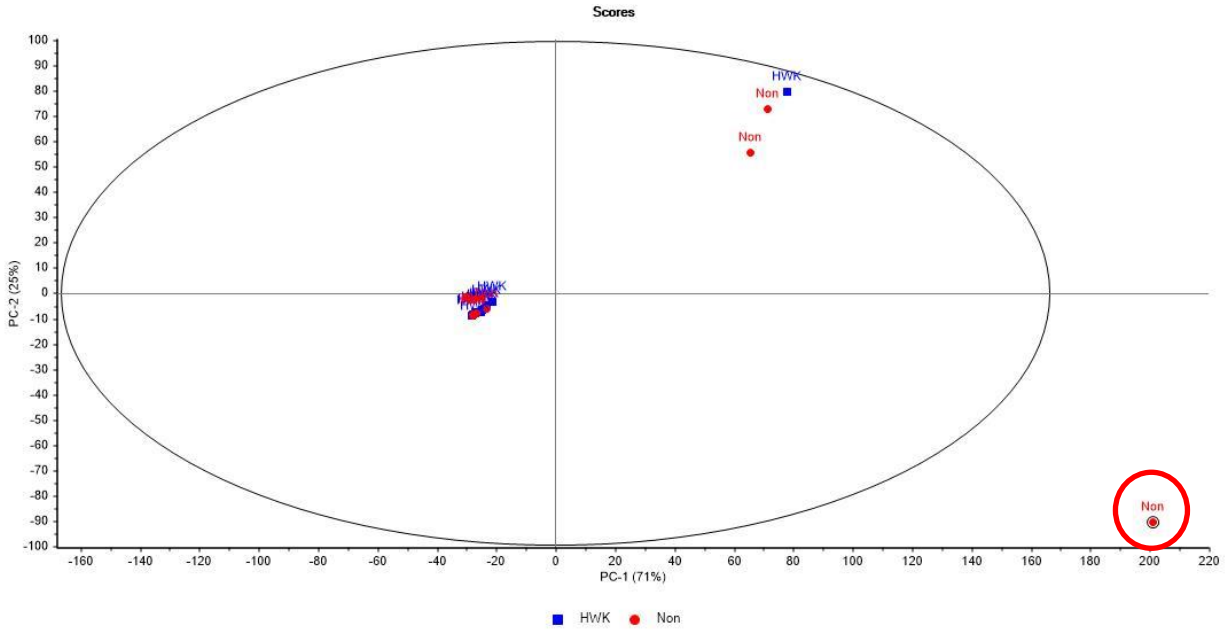


Figure 50: Variation between larva hot water killing method and hexane submersion shown in a PCA plot.

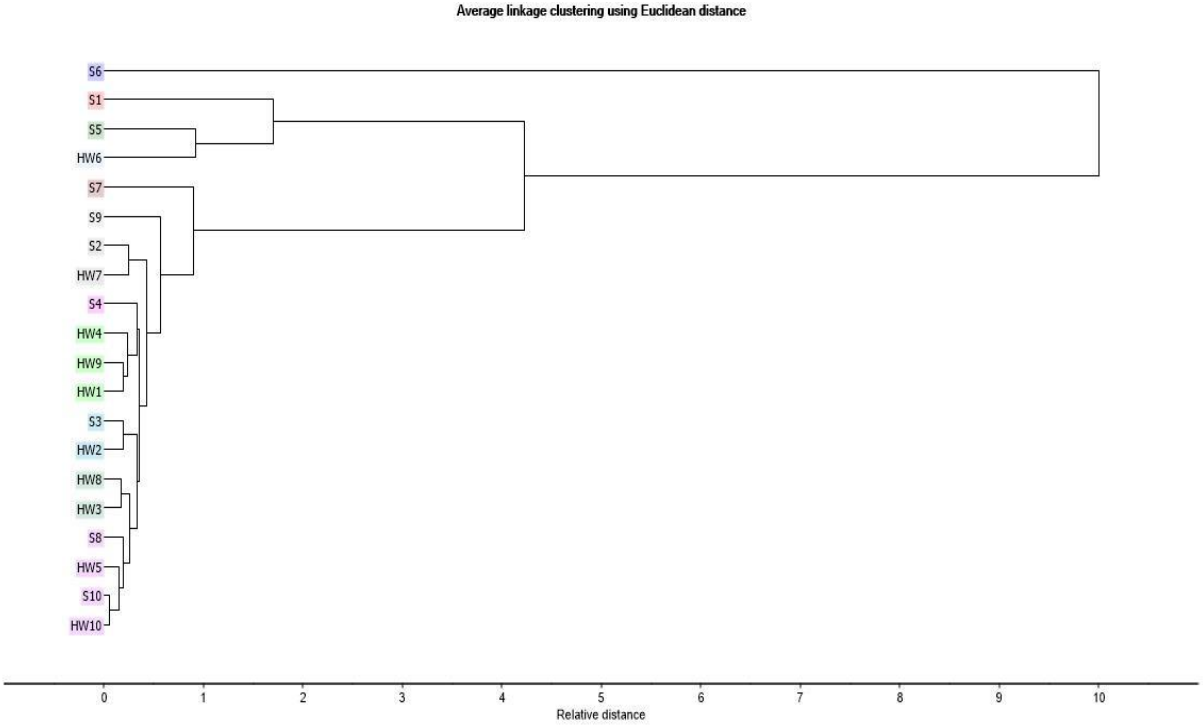


Figure 51: Correlation between different samples when analysing HWK, shown in a dendrogram. S= Samples immersed in hexane. HWK= Samples immersed in hot water and then hexane.

4.3.2 Conclusion

It can be concluded through the above analysis that the samples killed using hot water may show a slight difference in relation to the concentration of cuticular hydrocarbons present; however, the overall profile is consistent. This difference is not large enough that result of analysis would change, only a small percentage of the hydrocarbons were removed. External causes can encourage variances within the chromatograms, as can natural variation. Hydrocarbon analysis can still be carried out successfully using samples killed with hot water without any concern of cuticular hydrocarbon degradation or removal.

4.4 Comparison of the effect of freezing on GCMS Cuticular Hydrocarbon extraction in blowflies for forensic analysis

A second method often utilised during the sampling of larvae in order to kill the specimen using a recognised protocol is deep freezing. As soon after the collection as possible, samples should be placed into a deep freezer (ideally -20°C), for at least one hour [97].

This killing method can be applied to immature larval stages and also adult specimens. This is not a method exploited within this thesis; it is however, of interest in relation to the future use of cuticular hydrocarbon analysis and the effect that this technique may have on the hydrocarbons found on the cuticle of these insects should the specimen subsequently be submitted for cuticular hydrocarbon analysis. Should the technique enable no change in the cuticular hydrocarbons then larval samples killed in this manner following collection may be used for analysis without the need for collection of further specimens.

Ten third instar larvae were added to individual sample vials and placed into the deep freezer, which is set at -40°C for 2 hours. Larvae were then removed, allowed to thaw, then immersed in hexane, and extracted as detailed in Chapter 3. Ten larvae were also taken and killed by submerging them in hexane and analysed as above. Comparison was made between the two techniques focussing on the hydrocarbons.

4.4.1 Results comparing the hydrocarbon profile when freezing samples

Shown in Figure 52 is an example chromatogram from a sample killed by deep freezing compared by overlaying it with a sample killed by hexane submersion, this shows the concentration differences. Figure 53 show these chromatograms stacked to enable comparison of hydrocarbons present as opposed to concentration.

The profile shown in the samples does not appear to change in composition between the two sampling treatments. The concentration of the components is shown to decrease slightly with freeze killing. Again, it is not thought to be detrimental to accurate analysis. A difference in peak height is observed in peaks around 16.5 minutes and 19.5 minutes; these were both identified as not hydrocarbons.

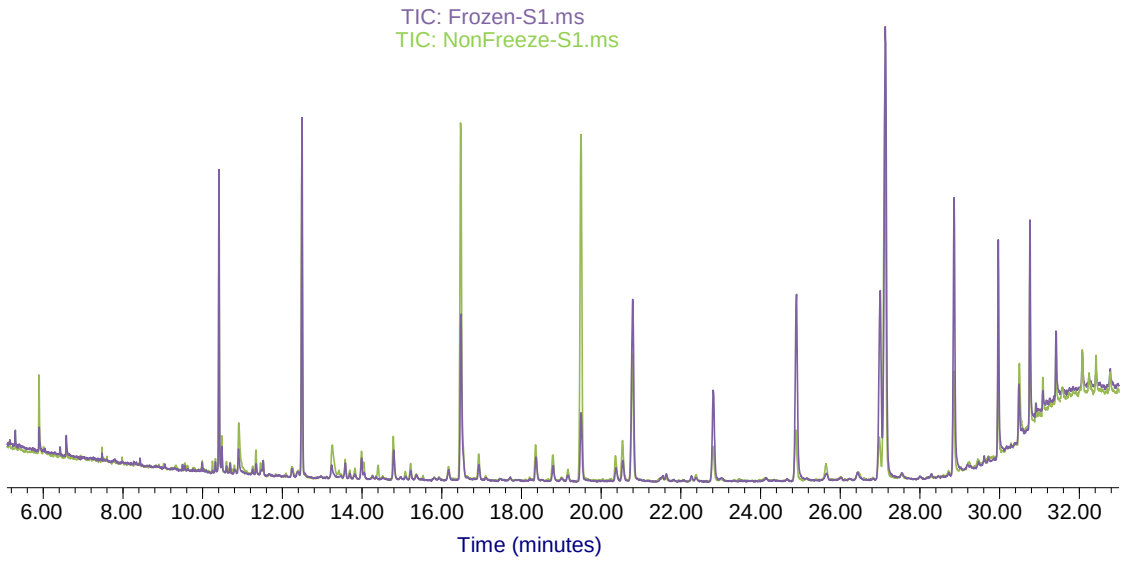


Figure 52: GC chromatogram showing the differences in Cuticular Hydrocarbons when comparing freezing as a larval killing method.

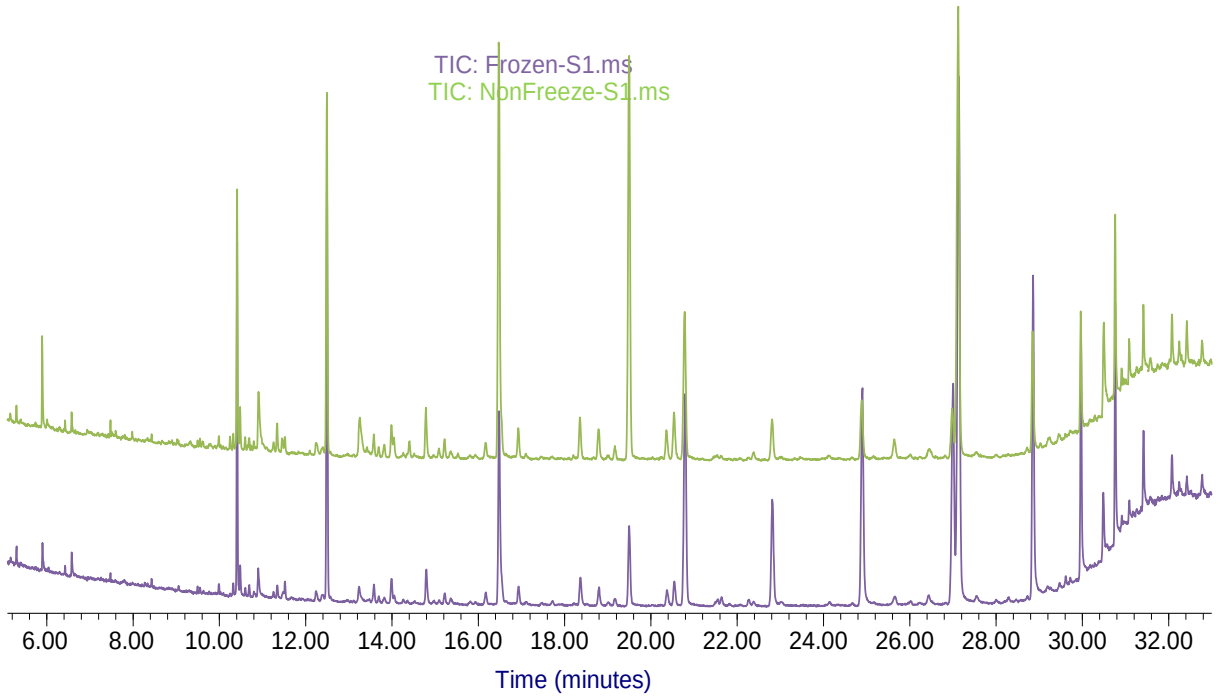


Figure 53: Stacked GC chromatogram showing the differences in Cuticular Hydrocarbons when comparing freezing as a larval killing method.

Chromatograms from both treatments were also analysed using Principle component analysis; PC 1 and PC 3 are plotted in Figure 54, together explaining 92% of the population variance. The samples did cluster together but not as tightly as seen previously. Within this cluster, samples did not show a pattern. This proved that differences were not only minimal but also not related to the treatment but instead likely to be due to natural variation.

Again, a dendrogram was used to explain the variation from the PCA further; this is shown in Figure 55. Sample 6 was shown to be significantly different but other samples were relatively similar.

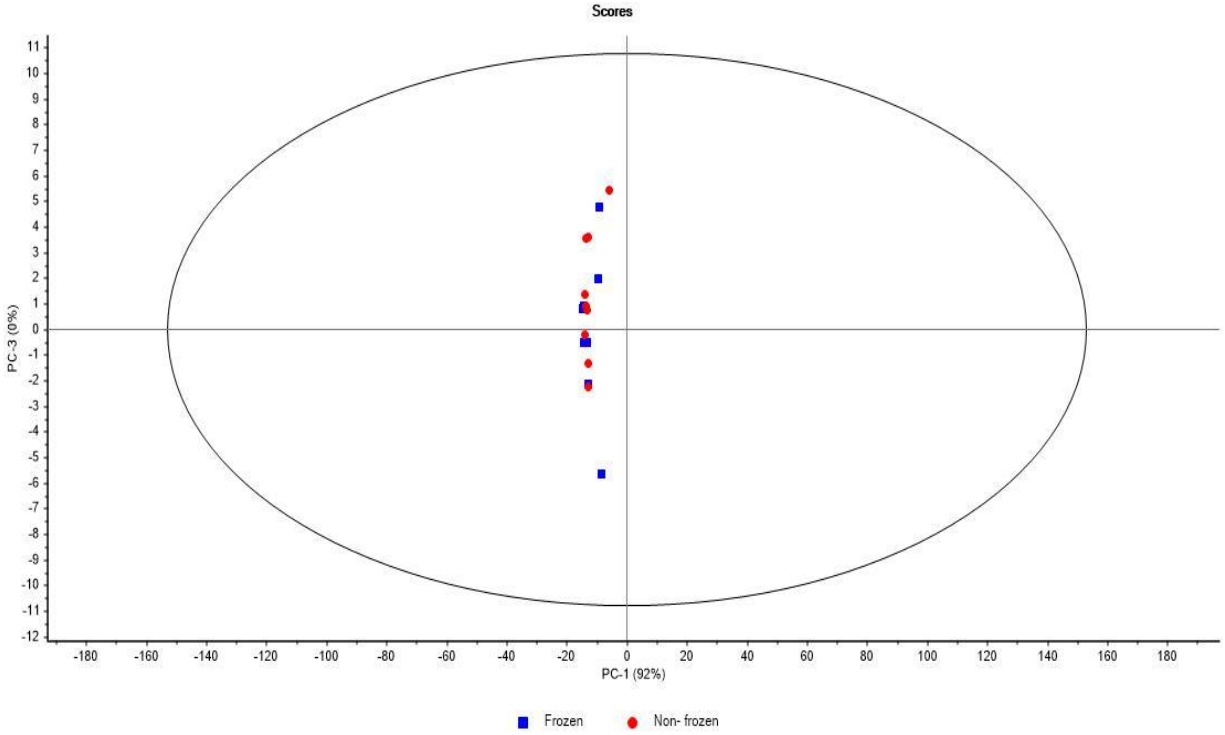


Figure 54: Variation between larva deep freeze killing method and hexane submersion shown in a PCA plot.

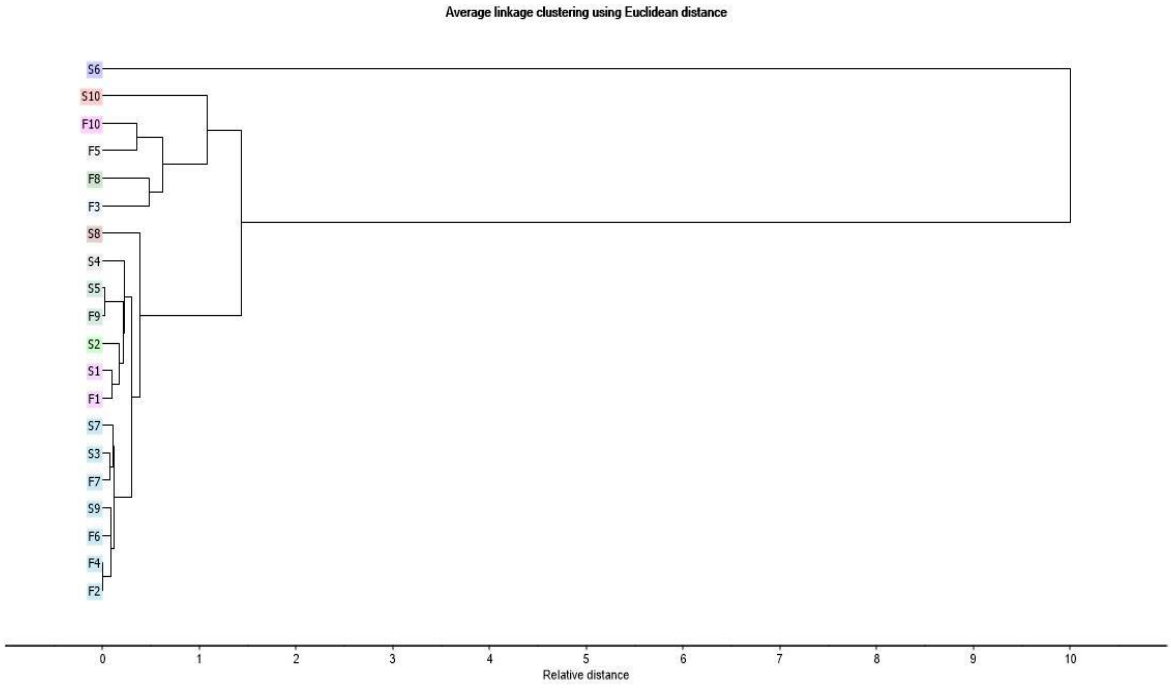


Figure 55: Correlation between different samples when analysing Freeze killed larvae shown in a dendrogram. S= Samples showing hexane submersion. F= Samples showing deep freeze killing.

4.4.2 Conclusion

It can be concluded that samples killed using the deep-freeze method can be used for cuticular hydrocarbon analysis as the hydrocarbons present do not show significant variation. The hydrocarbon profile is not degraded using this method and additional collection of samples would not be necessary for analysis.

4.5 Determination of solvent amount used for extraction of cuticular hydrocarbons

During the usual hydrocarbon extraction method, the sample is extracted using hexane as the solvent. After sufficient extraction time is allowed, the sample is run through a pre-wetted (with hexane) silica column to remove polar contaminants. At this point, a further volume of hexane is also run through the column to push through the desired components (hydrocarbons). It is hypothesized that increasing this volume of solvent may encourage a higher concentration of hydrocarbons. This was researched by completing the hydrocarbon extraction with three different volumes of solvent, the usual 500 microliters, 1 ml and 1.5 ml, effectively doubling and tripling the original amount. All samples are finalised by drying under a stream of nitrogen before adding 30 microliters and transferring the sample to an insert for analysis with GCMS. All sampling was carried out as explained in chapter 3, with the exception of solvent amount.

4.5.1 Results of determination of solvent volume for hydrocarbon extraction

A higher abundance of cuticular hydrocarbons was shown in the 1 ml and 1.5 ml extractions when compared with the 0.5 ml extracts, which is to be expected. Concentrations of hydrocarbons did not show significant enough improvement with 1.5 ml when compared to 1 ml extracts to permit the extra time required for drying the sample to completeness under nitrogen. A comparison of the chromatograms is shown in Figure 57; peaks circled were highlighted as noteworthy differences but were shown to not be hydrocarbons. Results were then analysed further using Principle component analysis where PC 1 and PC 2 were plotted describing 69% of the population variance (Figure 56). There is some overlap shown, however, samples extracted using 0.5 ml of hexane group further towards the top of the PCA plot and samples extracted with 1 ml and 1.5 ml grouped further towards the bottom of the plot.

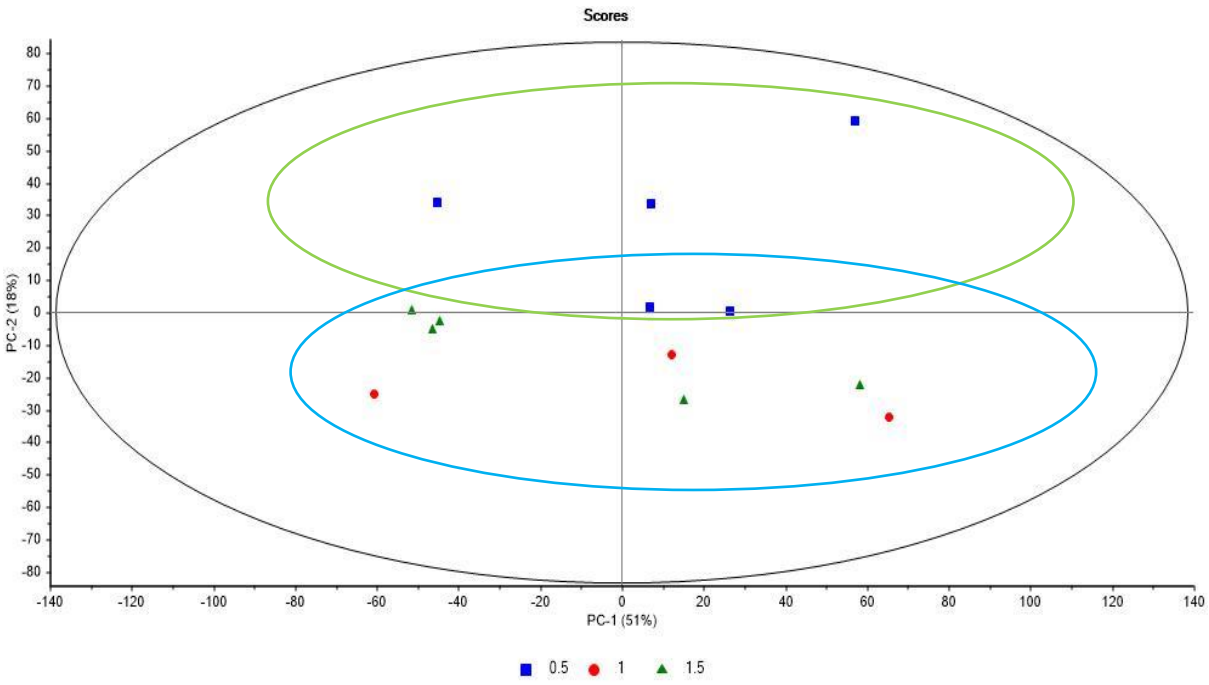


Figure 56: PCA plot showing differences observed when comparing solvent amounts during hydrocarbon extraction.

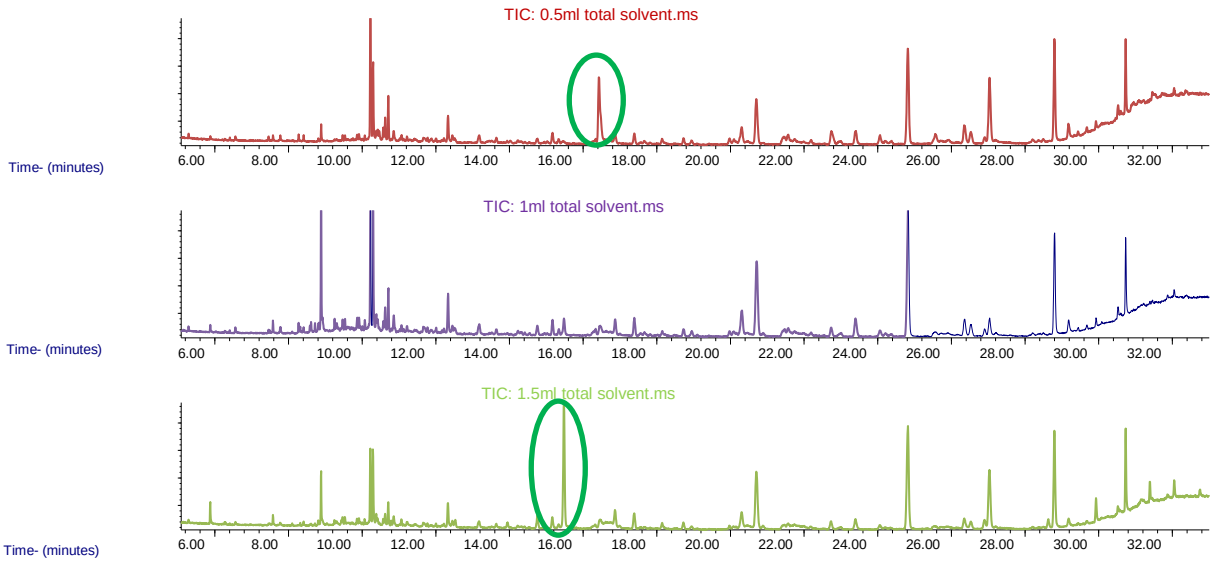


Figure 57: Three example chromatograms showing results of extracting samples with different solvent amounts.

4.5.2 Conclusion

Better extraction of hydrocarbons is beneficial to accurate cuticular hydrocarbon analysis, especially due to the small concentrations present. Using either 1ml or 1.5ml of hexane during the extraction process enables maximum extraction and subsequent sample analysis of a larger concentration of cuticular hydrocarbons. 1ml was selected for future extractions to encourage the development of this method but not to extend sampling time with excess hexane to dry down.

4.6 Comparison of Cuticular Hydrocarbon profile of blowfly over time after death

At present, there are no studies concerning the use of hydrocarbons to analyse and age dead flies found at a crime scene. This is of particular interest for any indoor scenario where blowfly would be unable to leave. This research focused on extracting hydrocarbons from the cuticles of the forensically important blowfly *C. vicina*, over time to characterise any chemical profile changes. It also provided a great basis to initiate part of the research focussed on the use of cuticular hydrocarbons.

Initially blowflies were collected as they died and packaged in enclosed petri dishes. The date of collection was noted, and samples were taken at intervals to analyse. These samples were kept at a constant temperature and extracted at five-day intervals across a three-month period. Chromatograms were then analysed to determine differences. The cuticular hydrocarbon profiles of each individual sample was analysed as shown in Chapter 3.

4.6.1 Results when comparing Cuticular Hydrocarbon profiles after death

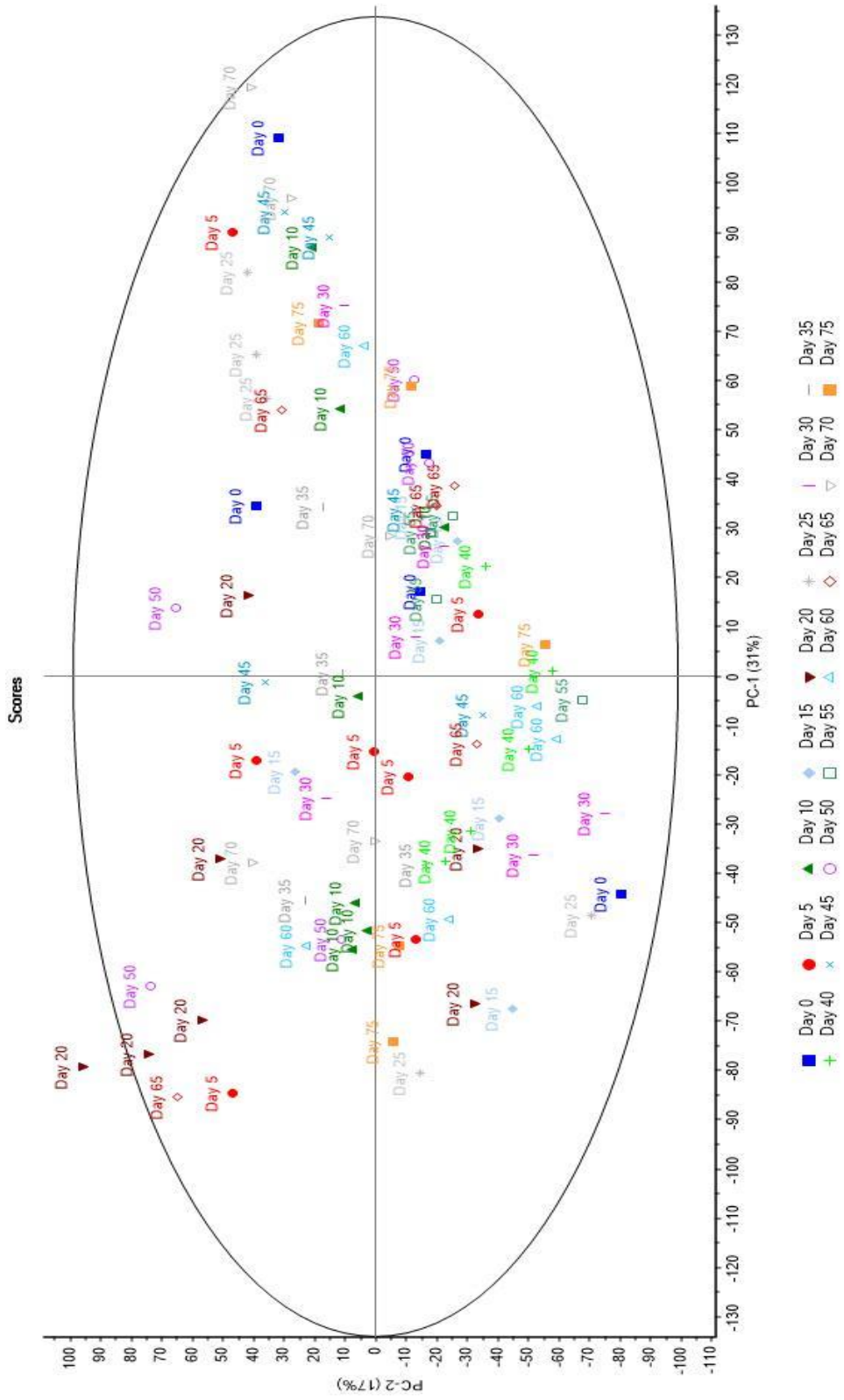


Figure 58: PCA plot showing an overview of the cuticular hydrocarbon profiles after blowfly death.

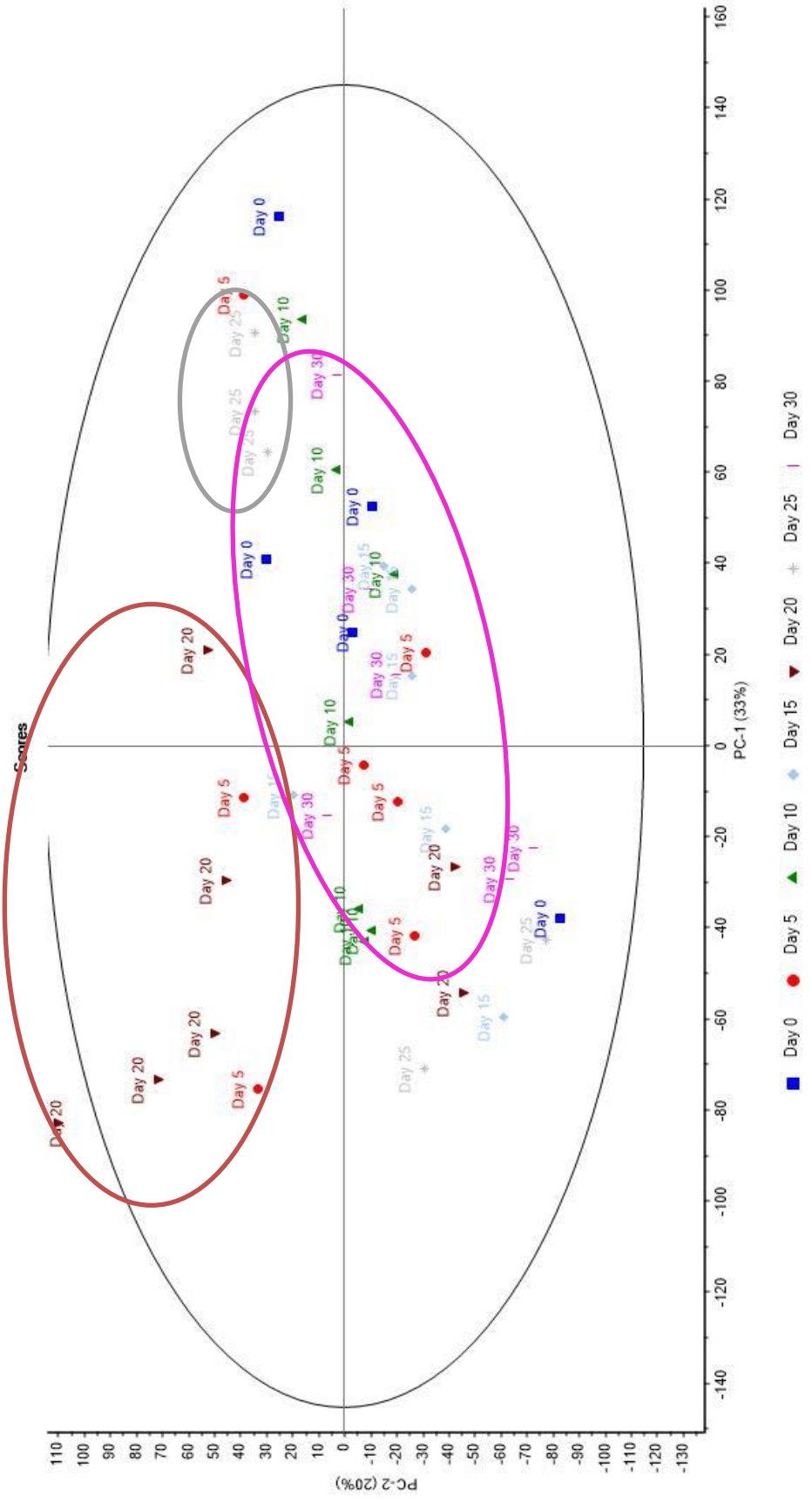


Figure 59: PCA plot showing day 0 to 30 of the cuticular hydrocarbon profiles after blowfly death.

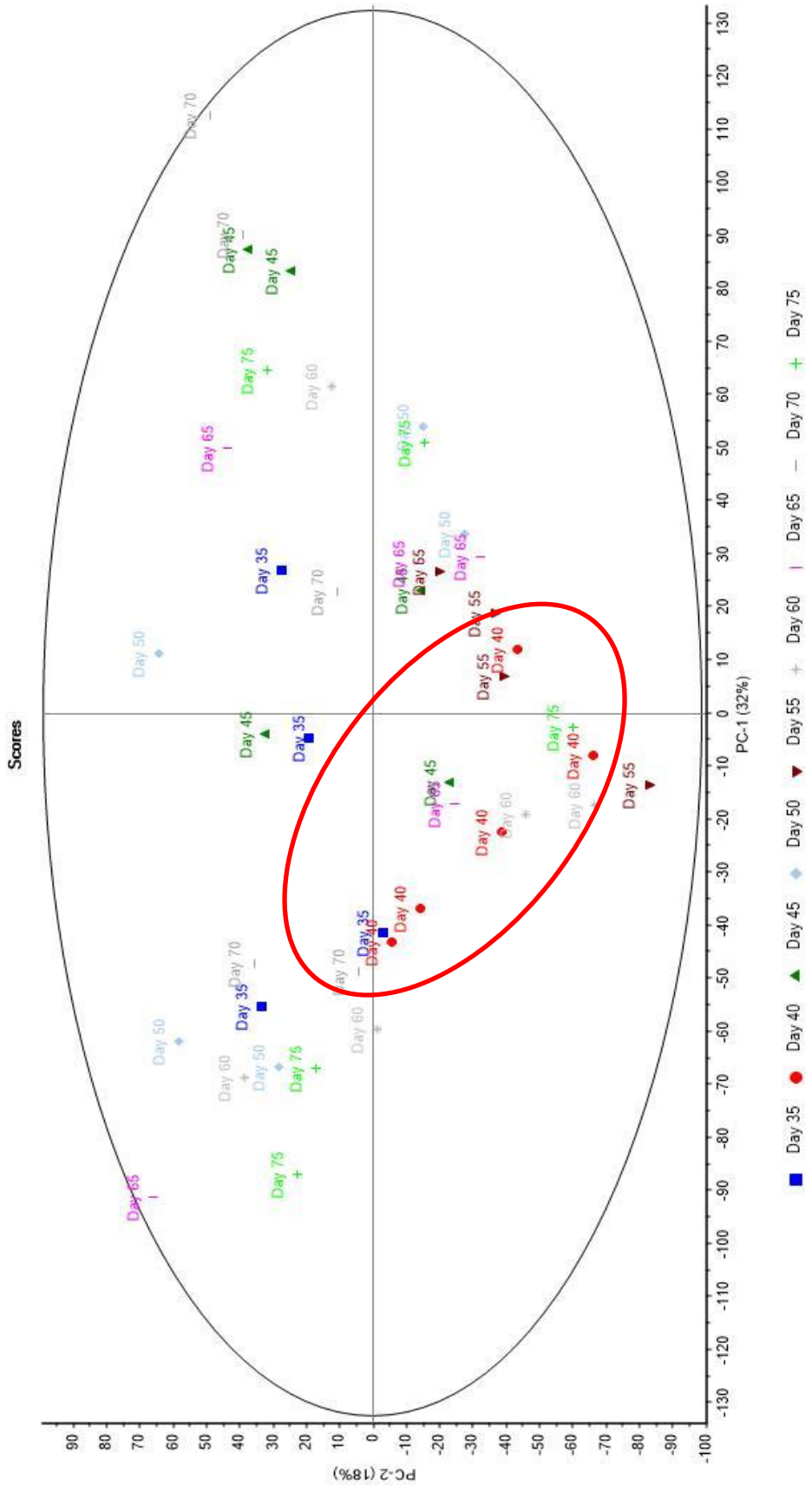


Figure 60: PCA plot showing day 35 to 75 of the cuticular hydrocarbon profiles after blowfly death.

4.6.2 Discussion and Conclusion regarding the analysis of dead blowfly hydrocarbons

Following the analysis of this preliminary data from the initial three months, it was noted that differing hydrocarbon profiles at death may mean the data could not be analysed in the manner intended. The initial results show that some compounds are detected in the earlier samples but are no longer present in later samples. The variation in compounds found in the samples will potentially help to age the dead blowfly once more information is acquired.

The principle component plots do not show much clustering at the beginning with the early samples but then it starts to stabilize, and the clustering can be more clearly seen. This is seen more clearly in Figure 6 and Figure 7 as a breakdown of the samples is shown. This could potentially be due to the hydrocarbon profiles containing such a large amount of variation when the blowflies die (as age at time of death of fly is not known). We have no information on the history of the fly before it died and as previous research has shown [234]; the hydrocarbon profiles do change with the age of the fly.

In Figure 6, Day 20, 25 and 30 can be vaguely clustered and in Figure 7 day 40. This analysis is by no means conclusive and requires further research to determine the nature of any changes observed. The current method analyses only the area of chromatogram where cuticular hydrocarbons are analysed. It could be improved with the addition of an internal standard for peak normalisation. If the chromatographic differences causing the change across the different time points are minor, then it may have been beneficial to focus on individual hydrocarbons as determined using a Principle component analysis.

If time had allowed, it would have perhaps been useful to re-run the experiment with a population of blowfly of known age which were then killed all at the same time, perhaps using dry ice to temporarily cause the blowfly to be immobilised and then place them into the deep freezer. Repetition of this experiment would provide results that are more conclusive.

4.7 Testing of drug derivatisation method

To enable successful analysis of drugs with GC-MS for future research, derivatization must be considered. The composition of Novel Psychoactive Substances (NPS) is unpredictable and unknown. Derivatization reactions enable the transformation of an analyte for better detection on the GC. The derivative is likely to have a similar or closely related structure [282].

Three derivatising agents are considered. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) is a silyl reagent enabling a silylation reaction. It is used to enhance the performance of the GC by increasing the volatility of the analyte and decreasing surface adsorption[283]. A derivative produced with a silylation reagent is generally more volatile, less polar and more thermally stable.

Trifluoroacetic anhydride (TFAA) and Heptafluorobutyric acid (HFBA) are acylation reagents which work by converting compounds with active hydrogens to encourage a derivative which is stable, highly volatile and has a good peak shape[283].

Samples of drugs of interest, shown in chapter 3, were derivatized separately using each of the three reagents specified and then chromatograms were analysed to determine the

outcome and success levels. This then helped to define the derivatising reagent of choice for future experimentation regarding drug analysis.

BSTFA reagent was diluted in a solution with ethyl acetate in a ratio of 5:1, TFAA and HFBA were used as they were.

For BSTFA and HFBA, the samples were dissolved in 50 µl of ethyl acetate, 50 µl of the reagent was then added to the sample. The samples were then heated to 70°C for 30 minutes and then allowed to cool to room temperature before placing under a stream of nitrogen to dry completely. 50 µl of ethyl acetate was then added to the sample and the sample was transferred to an insert before analysis with GCMS.

For TFAA, samples had 100 µl of reagent added to them, which were then heated to 80°C for 10 minutes before drying with nitrogen and reconstituting with 50 µl of ethyl acetate in an insert, which was then run on the GC-MS.

The oven temperature program for analysis of drugs and internal samples was as follows: hold at 100 ˚C for 3 minutes then ramp to 140 ˚C at 20 ˚C/min and hold for 2 minutes. Ramp again to 150 ˚C at 5 ˚C/min and hold for 2 minutes. Ramp to 160 ˚C at 5 ˚C/min and hold for 5 minutes. Finally, ramp to 250 ˚C at 20 ˚C/min. Elution was carried out at 1mL/min with helium.

A selection of results is shown in the figures following.

4.7.1 Results of drug derivatisation

5APB shows clear narrow peaks with no peak tailing when derivatized using HFBA. Chromatogram quality is reduced with BSTFA and TFAA and peak tailing is also visible. A shows TFAA derivatisation, B shows BSTFA derivatisation, C shows HFBA derivatisation and D shows the non-derivatized drug.

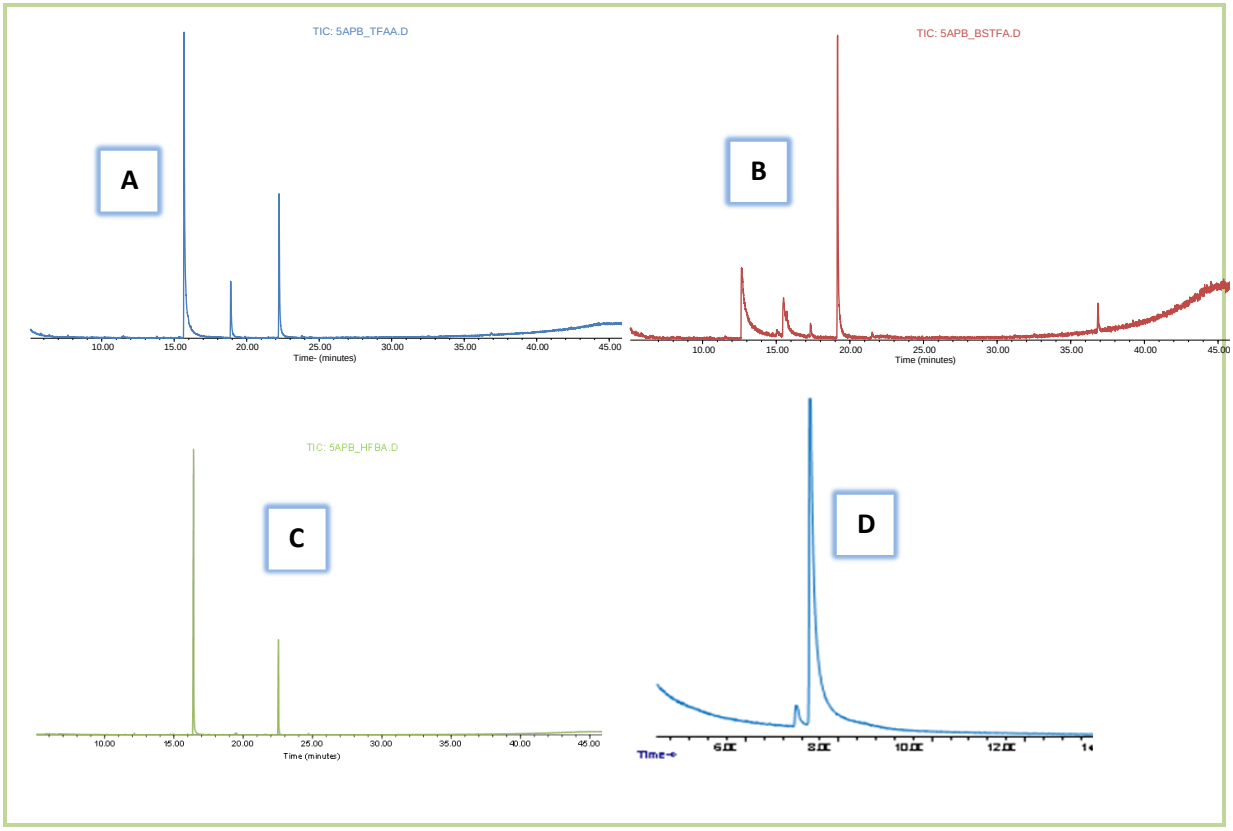


Figure 61: Chromatograms showing three derivatization trials of 5APB compared to the drug non-derivatized.

5EAPB appear to show a clear analysis for all reagents, slight peak tailing is shown using BSTFA.

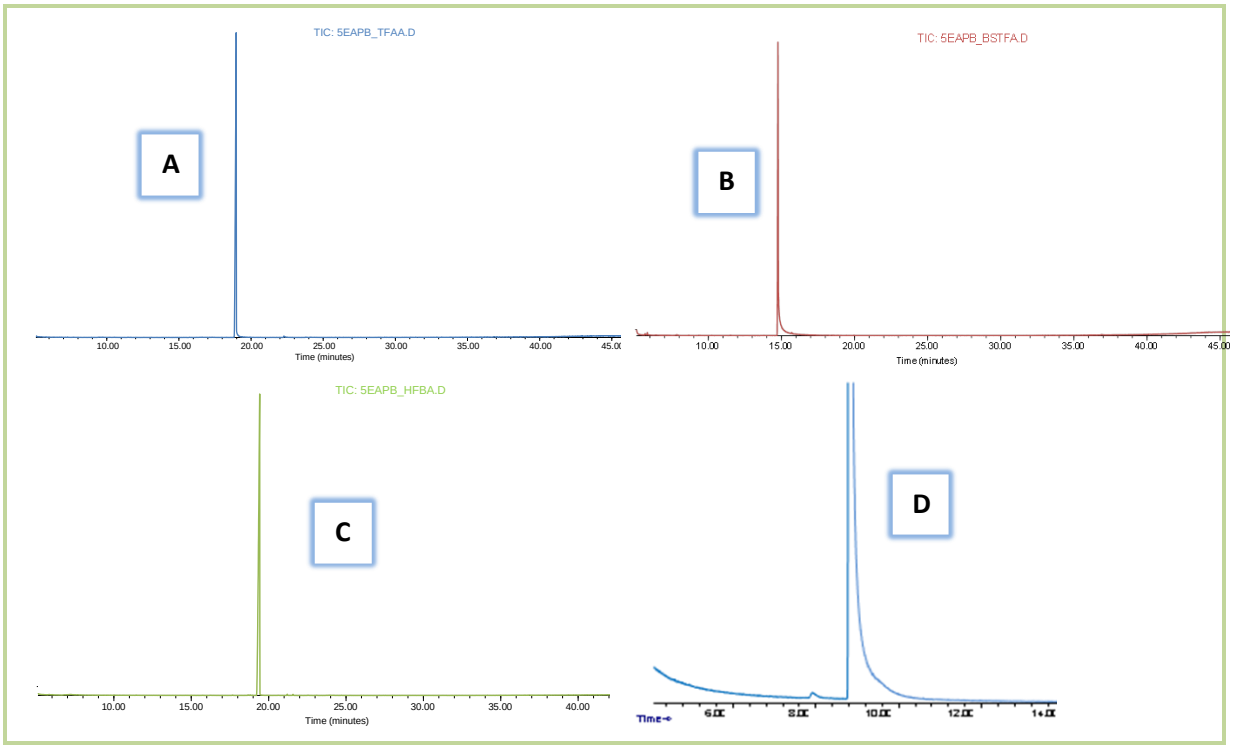


Figure 62: Chromatograms showing three derivatization trials of 5EAPB compared to the drug non-derivatized.

6+5 APB does not work well with BSTFA, whereas TFAA and HFBA return clear well defined peaks.

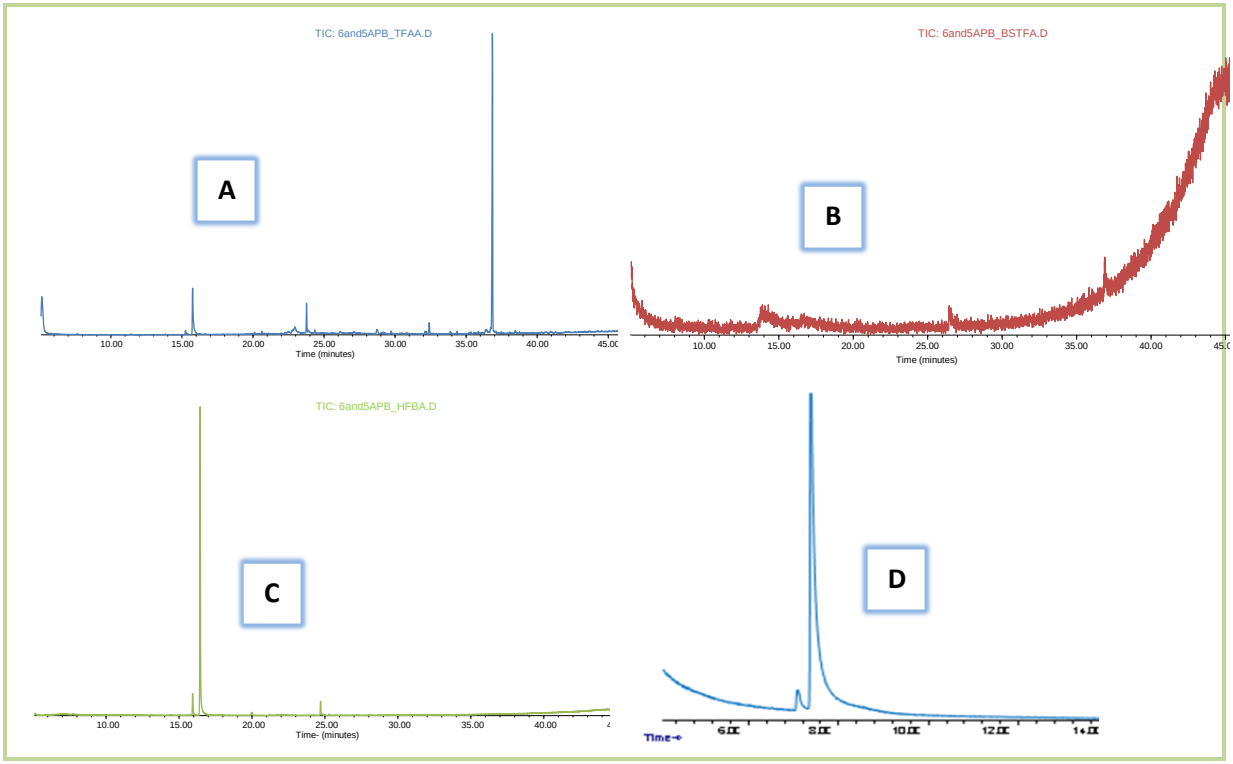


Figure 63: Chromatograms showing three derivatization trials of 6+5APB compared to the drug non-derivatized.

Benzofury does not derivatize well using BSTFA or TFAA, again clear peaks are shown using HFBA.

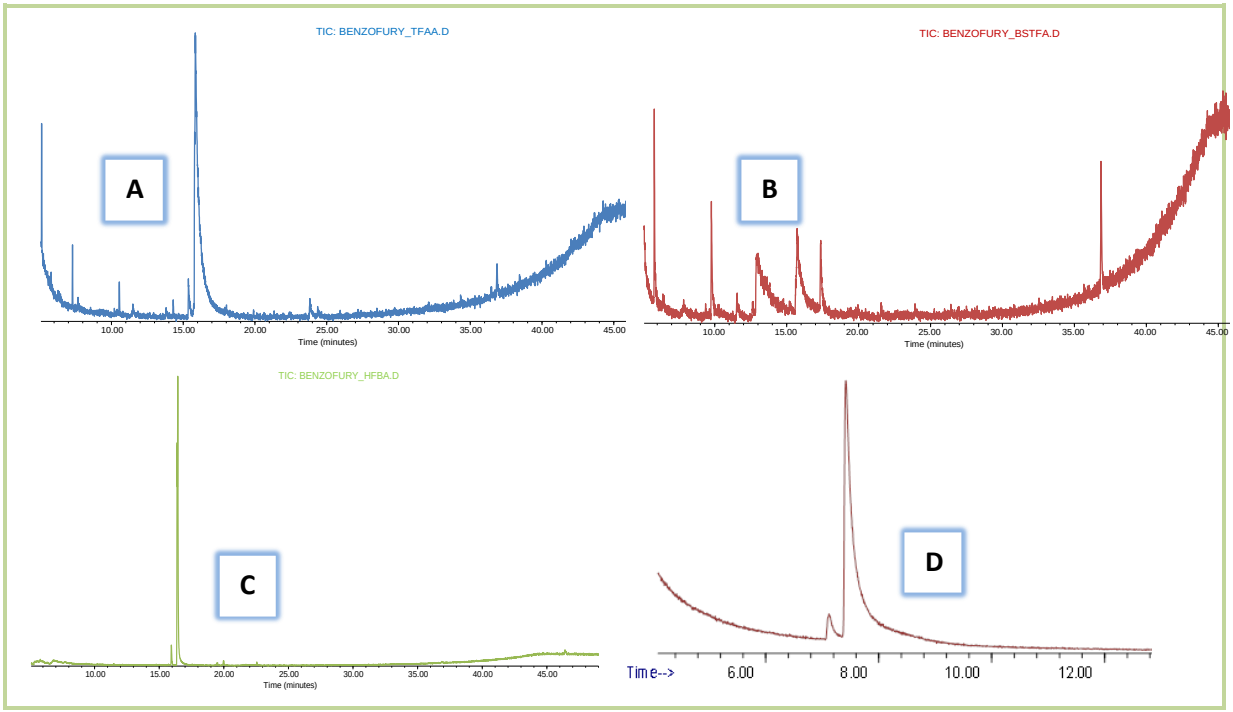


Figure 64: Chromatograms showing three derivatization trials of Benzofury compared to the drug non-derivatized.

Blow appears to react well to all derivatization agents but slight peak tailing is observed when using TFAA and BSFTA.

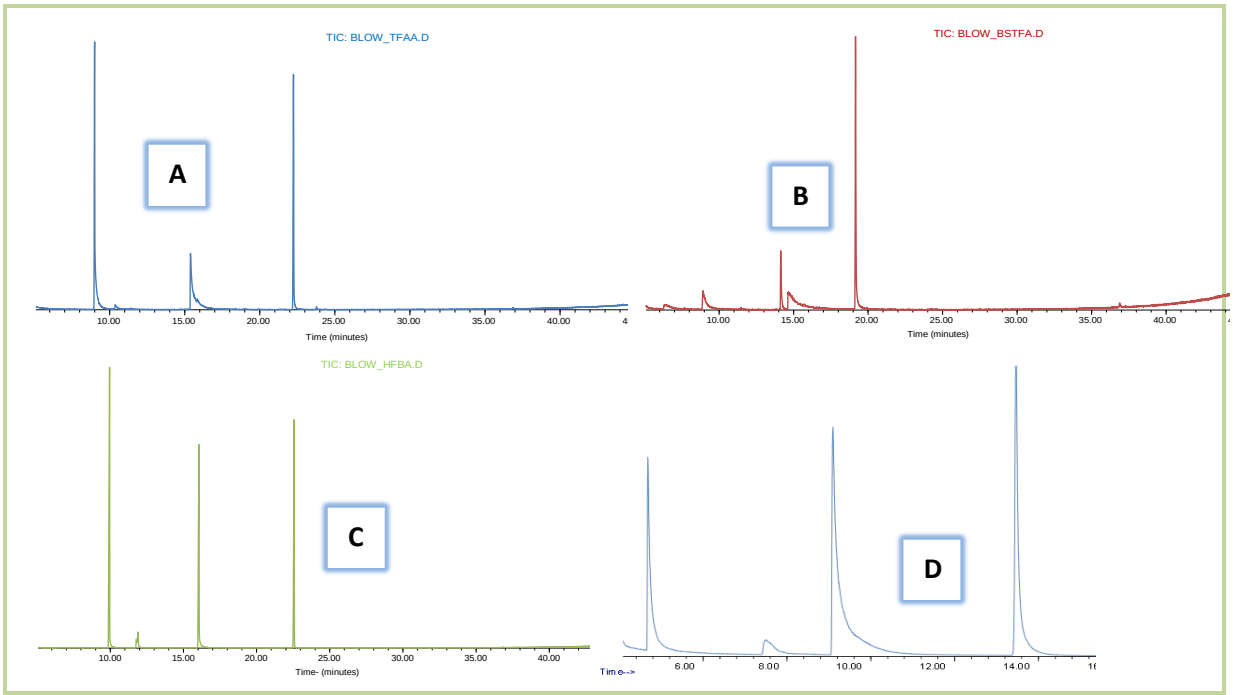


Figure 65: Chromatograms showing three derivatization trials of Blow compared to the drug non-derivatized.

MDA does not work well with BSTFA; there is little difference between TFAA and HFBA derivatives.

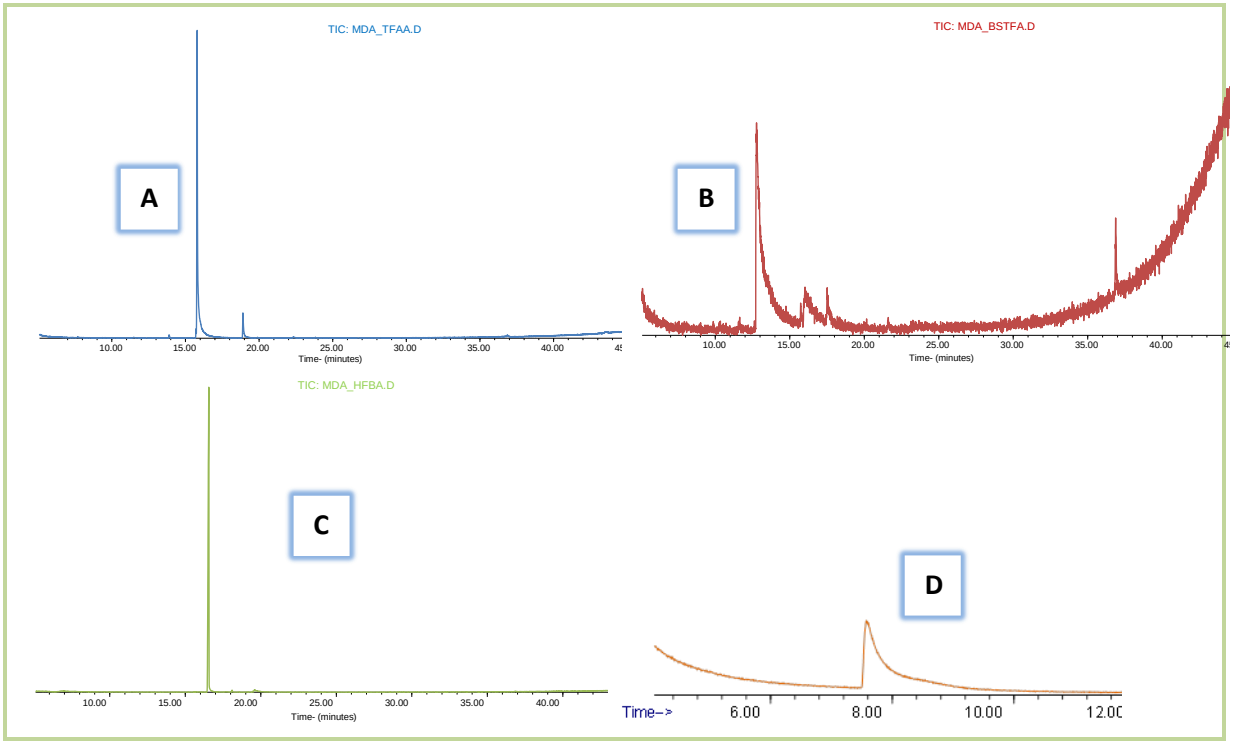


Figure 66: Chromatograms showing three derivatization trials of MDA compared to the drug non-derivatized.

4.7.2 Conclusion

BSTFA does not appear to react well with amphetamine structures. HFBA performed overall the best, across all analysed compounds giving a good peak shape and abundance. Given the unpredictable nature of NPS, therefore this is the obvious choice for use as a derivatising agent.

4.8 Solvent Choice for drug analysis

For later research, it is important to ensure the novel psychoactive substances (NPS) with unknown composition were soluble in the chosen solvent. Analysis was therefore performed with two different solvents; Ethyl Acetate and Dichloromethane. These samples had been derivatized as per the method predetermined by previous experiments. Each drug was also sampled with Dichloromethane as the solvent but in a non-derivatized form for comparison. Visibility of drug composition was then determined through analysis of the resulting chromatograms.

4.8.1 Results of solvent selection for drug analysis

A selection of chromatograms is shown in Figure 67, Figure 68, Figure 69 and Figure 70.

Components of the NPS of concern within the scope of this research are clearly visible with both Dichloromethane and ethyl acetate for all drugs, and as expected the peak positions remain the same. As a comparison, the compounds with dichloromethane as a solvent but are un- derivatized have different peak positions, as expected. Concentrations remain very similar when the two solvents are compared.

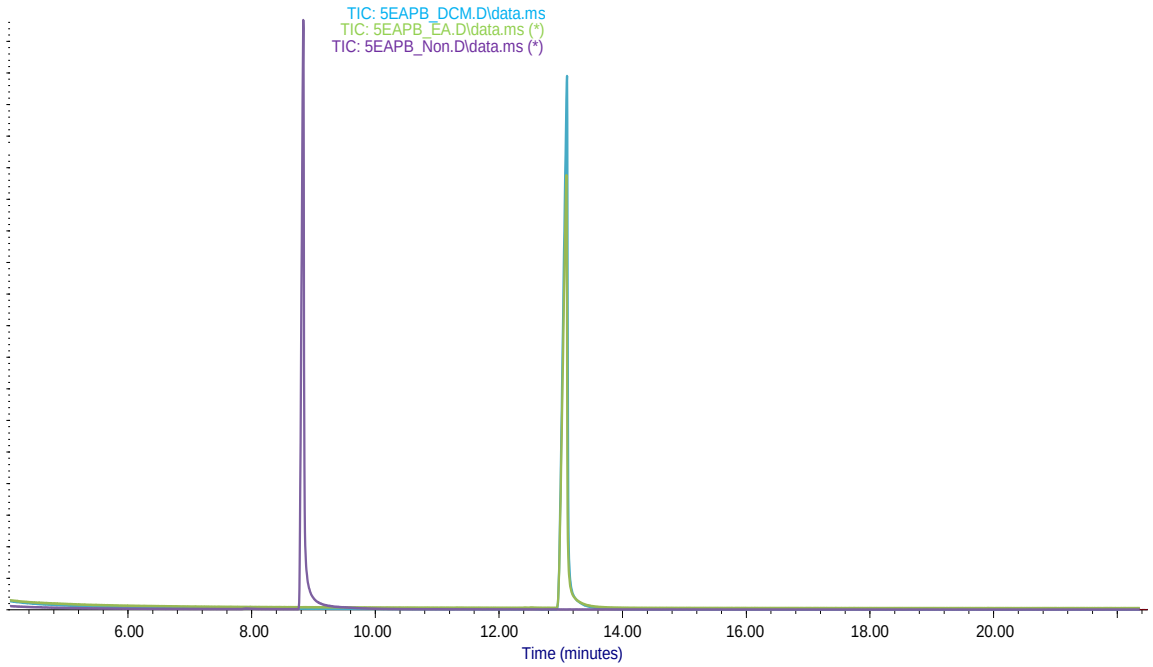


Figure 67: Chromatogram overlay of analysis of 5EAPB with different solvents.

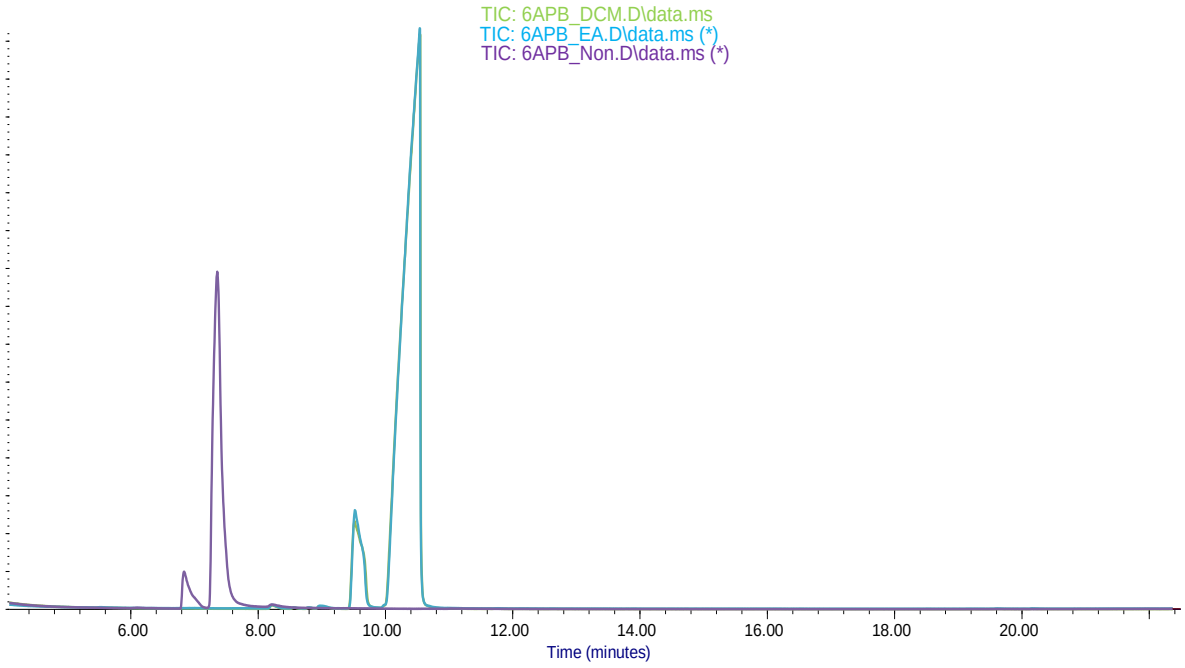


Figure 68: Chromatogram overlay of analysis of 6APB with different solvents.

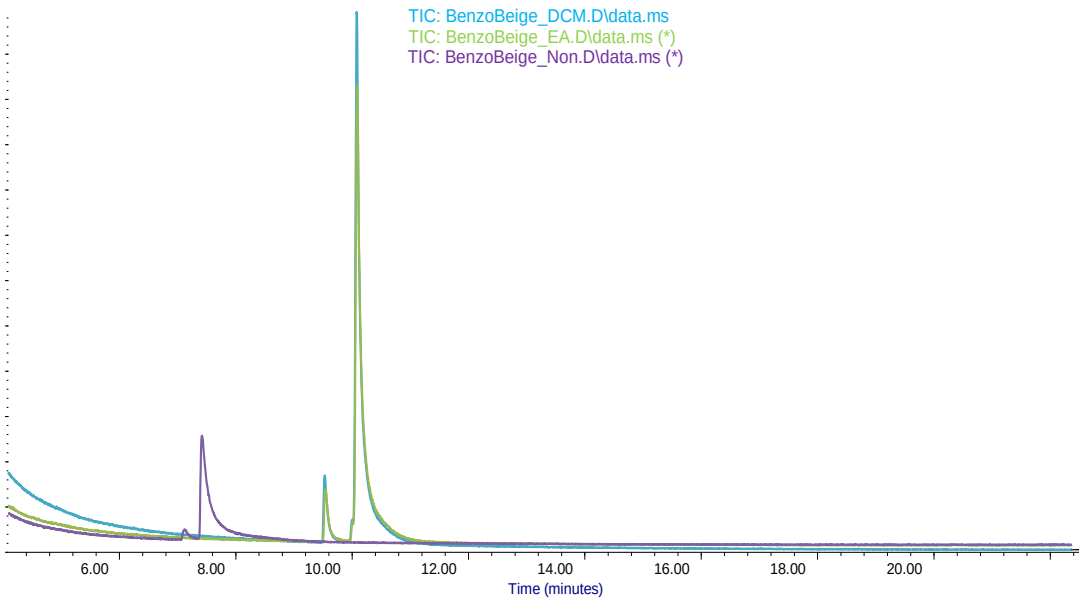


Figure 69: Chromatogram overlay of analysis of Benzofury Beige with different solvents.

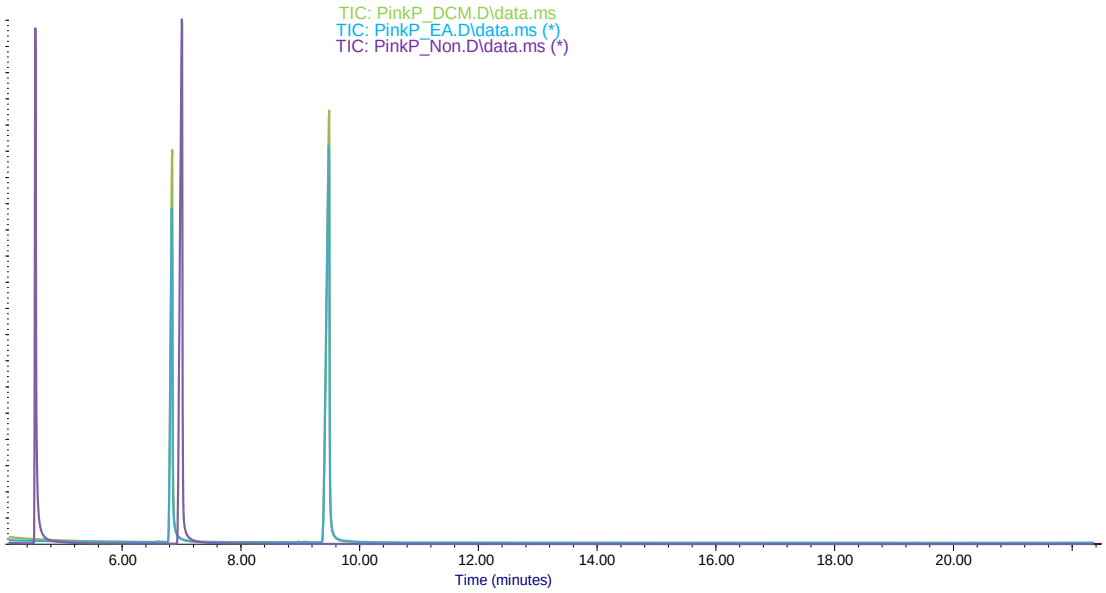


Figure 70: Chromatogram overlay of analysis of Pink Panther with different solvents.

4.8.2 Conclusion

It can be concluded that for the analysis of novel psychoactive substances within this project, either Ethyl acetate or Dichloromethane can be utilised with no detrimental effect due to insolubility.

4.9 Diets and Agar

4.9.1 Observations of initial diets

Initially animal tissue was spiked with a drug solution and then the solvent evaporated. As part of the preliminary work, it was necessary to ensure that the flies would show interest and feed from the diet, which has been spiked in this manner. Therefore, a solution of paracetamol at 1000 ppm was added to mincemeat and mixed thoroughly; this was then left under an extractor in a fume hood until all solvent had evaporated. The diet was then introduced into a colony of blowflies, purely to see if they would show interest and if there were any obvious behavioural changes. No quantification was done at this point.

Initial observations of preparing drug diets in this manner showed the animal tissue to be desiccated with a hard 'crust' upon presentation to the fly colony. This was not as attractive or palatable to adult flies, who are attracted to a source of food often as a result of necrotic odour, defined as the odour associated with dead tissue. There was also a concern using this method that an amount of solvent may remain within the diet, this would not allow testing of the individual drug present, as there may be other variables potentially causing change.

Mixing a powdered drug with pureed meat or minced meat was also tested, this method was not considered to produce a homogenous diet, and this would introduce variation. The use of a mixer to distribute the drug as evenly as possible within the diet could also become a source of potential contamination.

The necessity of an artificial diet to overcome these issues was realised.

4.9.2 Artificial Diets

In order to successfully evaluate the effect of NPS on blowfly development, a method to enable consumption is required. Research shows the use of animal tissues [23], [52], [73], [102], [125], [156], some where drug addition is post death and other showed the drugs were administered prior to death. This raises two issues for consideration; firstly, ethical approval for studies of this nature are both lengthy and uncertain, further thought must also be given to the sourcing of the animal model and administration of the drug prior to death. Secondly, the use of different animal tissues is shown to produce inconsistent data [284], tissue type is further discussed in chapter 1.

It was concluded that an artificial diet may overcome these barriers whist also enabling the addition of drugs homogenously, the exact composition will be known and therefore these variables controlled.

There have been numerous diet suggestions for different rearing requirements [155], [285]–[289], all of which were considered when addressing the requirements of this study; combining substances of known nutritional value to produce an homogenous and reproducible artificial diet which performs as well as animal tissues. These diets are especially good at reducing water loss to ensure longevity of the diet. The diet was not frozen and was made fresh as needed.

The ideal relative density suggested in research is 1 gram of diet per larvae [290] and hence diets were produced in two lots of 300 g amounts and 600 larvae were used. This should avoid the stress produced by competition for food.

4.9.2.1 Methodology concerned with preparing an artificial diet

The requirement of the research to incorporate substances of interest, in this case drugs, into the food source has identified the option of artificial diets.

Artificial diets are a heavily researched topic with many different suggested ingredients.

An experimental method was created to test the effect of different artificial diets on larval development for potential use in future experimentation.

Combinations of nutrient agar, defibrinated horse blood and liver were produced as detailed below; the source is detailed in chapter 3:

A) Nutrient agar
B) Nutrient agar with horse blood
C) Nutrient agar with liver and horse blood
D) Nutrient agar with liver
E) Liver (to act as a control)

Age of horse blood and liver were always kept the same so not to introduce a further area of variation.

Diets were created by adding boiling distilled water to nutrient agar at a ratio of 9 g per 187.5 ml (Figure 71), then adding blood (22.5 ml) and/or liver (90 g). The solution was mixed thoroughly and left to set before using Figure 71. Eggs were taken from a colony of blowfly, prior to hatching the equivalent mass of 40 eggs were added to each one of the five diets. The petri dishes containing the diets were then placed in an individual box with

sawdust underneath, so as to allow an appropriate substrate for post feeding larvae. Boxes were placed inside an incubator at 23 °C.

Larval behaviour was monitored, and wandering was noted. Ten larvae were randomly picked and weighed once a day. Length was not measured, so not to have any need to kill the larvae. Each set was observed for post feeding, pupariation and emergence rate.

Figure 71: Preparation of the agar diets and five initial diets used in preliminary artificial diet development.

4.9.2.2 Results of diet development

As shown in Figure 72, the diet with agar only did not perform well and larvae did not survive past the first day and no development took place, the nutrient value must have not been sufficient. With the addition of blood to the agar diet; survival rate increased, but not dramatically and certainly not within an acceptable level, larvae were also shown to be visibly very small throughout the duration of the experiment and difficult to source

due to this. Three of the diets showed promising results, the liver control, the agar mixed with meat and also the agar mixed with blood and meat. Agar and meat showed a very similar pattern to the control with a lower larval weight at day seven and earlier plateauing of the data is observed. The agar diet with blood and meat appeared to outperform all diets including the liver control. All diets were observed to monitor adult emergence. Agar and agar mixed with blood did not emerge; all others emerged as expected, with results consistent with the liver control population.

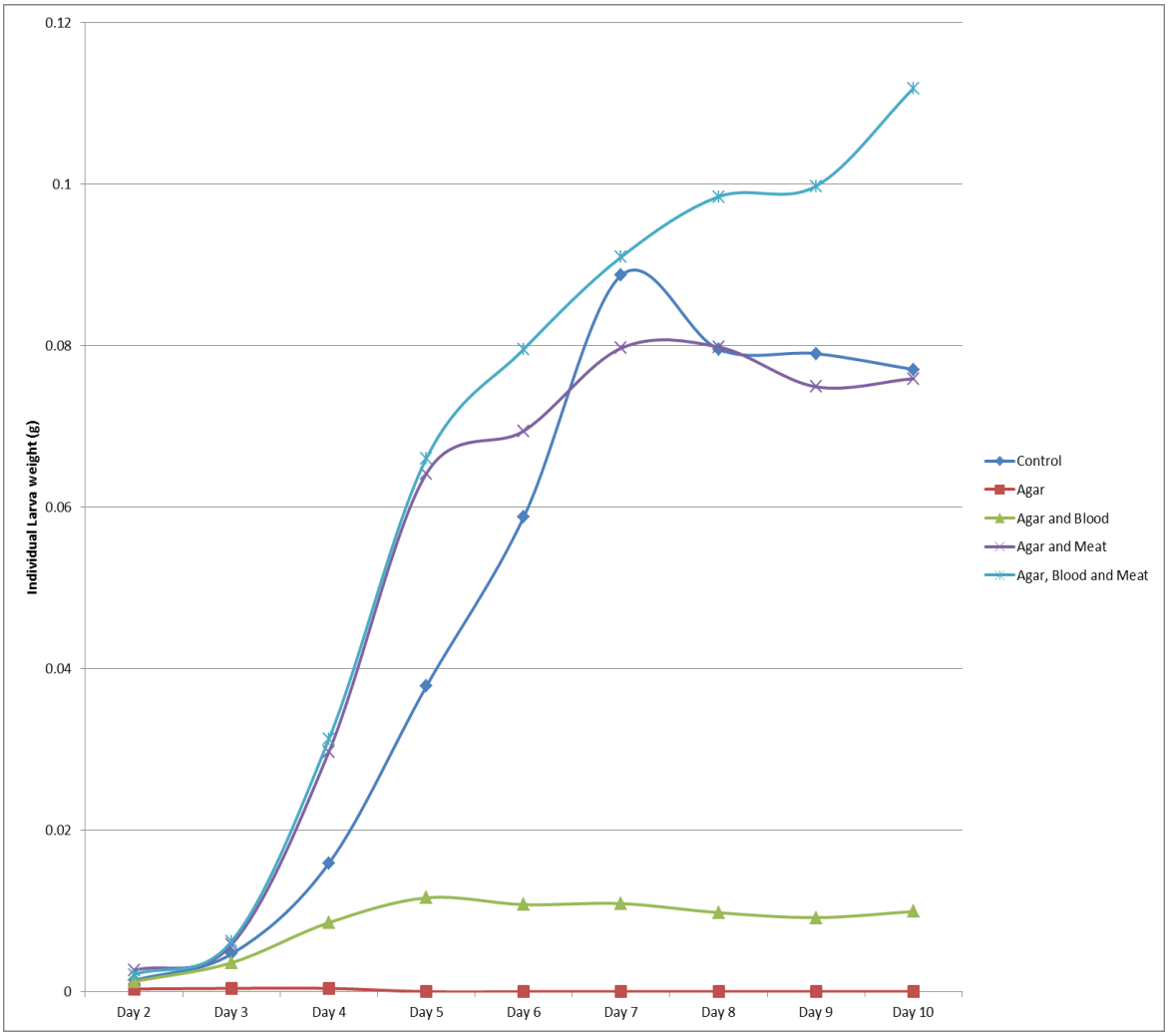


Figure 72: Results of diet development on average larval weight.

4.9.2.3 Diet improvements

Some research suggests the use of other components such as powdered milk and yeast. The results from the prior study suggested agar with meat and blood addition to be a suitable artificial diet. The following study was then performed to determine if the diet could be nutritionally enhanced.

A) As above (Agar with meat and blood addition)

B) As above with yeast (4 g)

C) As above with powdered milk (4 g)

D) As above with powdered milk and yeast (4 g and 4 g)

E) Liver (to act as a control)

4.9.2.4 Results of potential diet improvements

The line graph in Figure 73 shows further development of the agar diet. Performance must be equal to or above that of the control to ensure accurate developmental data in relation to drug affect. After further research, a number of additions were suggested to improve the diet. These were sampled, again against a liver control. All diets appeared to outperform the liver control and the diet showing largest larvae was agar with meat and blood, no further additions.

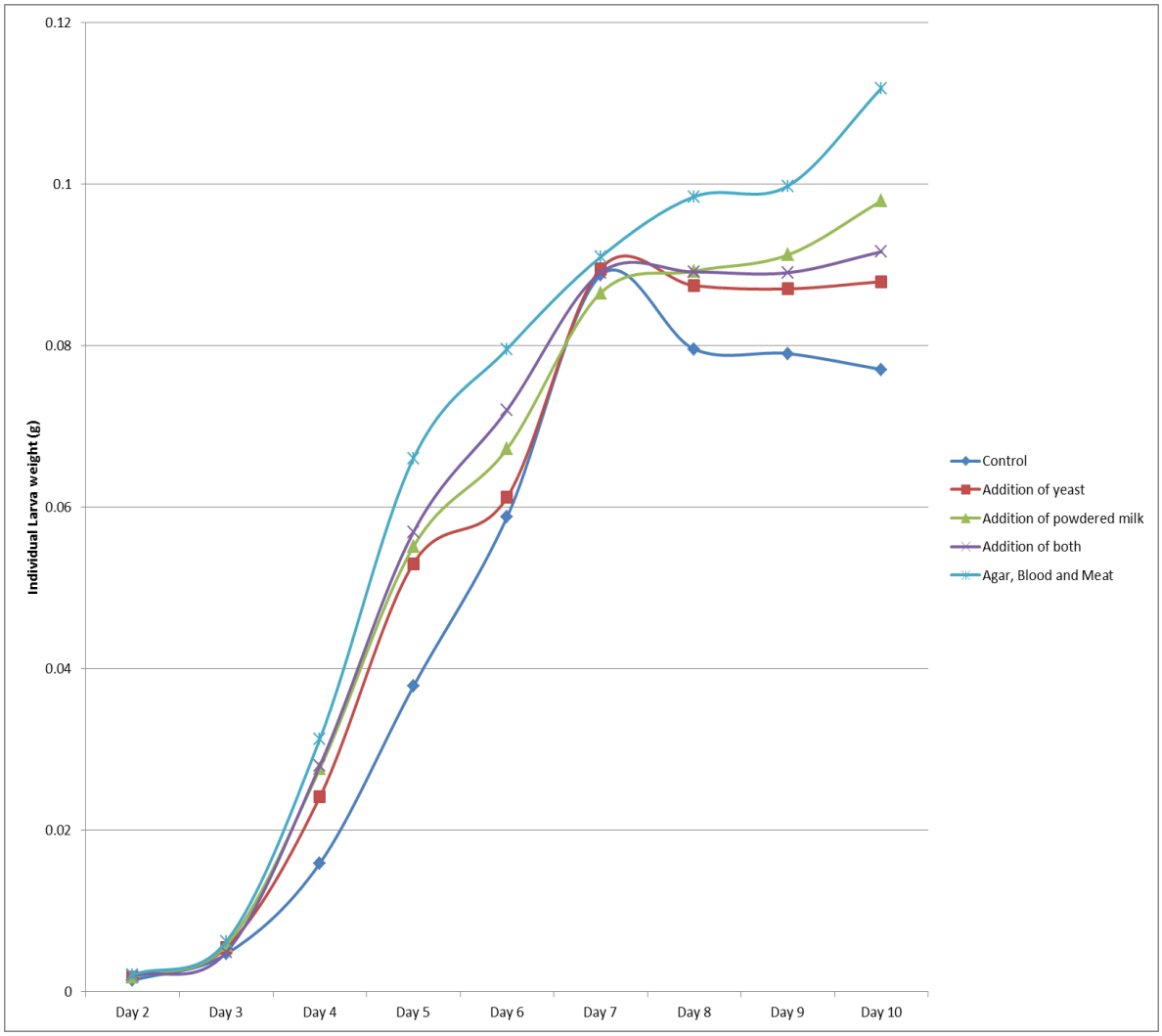


Figure 73: Average larval weights compared across the developed diets.

4.9.2.5 Conclusion

Further development would be outside the scope of this thesis, a diet performing better than the control has been developed and enables the addition of drugs homogeneously without the problems previously experienced. The diet was kept uniform other than the drug addition throughout this research so that the effect can be compared to the artificial control. The artificial diet to be used from now on is Agar with meat and blood added. It should be noted that the sudden increase in larval weight at day ten, experienced in the diet containing agar, meat and blood is unexplained.

4.9.3 Artificial diet with drug addition compared to meat with drug addition

An experiment was carried out to determine if any effect was seen during the comparison of the pre-determined artificial diet with the addition of caffeine and a minced liver diet with caffeine. A comparison should determine if the use of an artificial diet affects drug availability. Ten larvae were sampled daily using the methods already described, but now killed using hot water and length in mm was recorded. An average of the larvae sampled per day was taken and plotted for comparison in Figure 74. Both diets had the addition of caffeine at the same concentration, as the effect of the drug was not of interest in this study. It was hypothesized that measurements taken from the minced liver would vary more due to an uneven spread of the drug. The artificial diet however was thought to provide a more homogenous medium and therefore results per day should be less varied.

4.9.3.1 Results comparing larval development on artificial diet and meat diet with drug addition

Some noteworthy differences were shown when averaging the daily sample measurements as seen in Figure 74. Measurements from the drugged liver did vary more. Drug availability was affected by using a diet of minced liver; it is suggested that this method is not able to offer an even spread of the drug and larvae feeding will therefore differ in the contents of their diet that they are feeding on, some will ingest the drugs and others will not. It is also possible that an individual larva may find an area with drug present unpalatable due to the high concentration found there and move elsewhere where no drug is present. This could explain the decrease in larval length observed. This

could also be due to the nutrient value of the diet offered. Differences in average lengths are not largely significant; the agar does however consistently produce longer larvae.

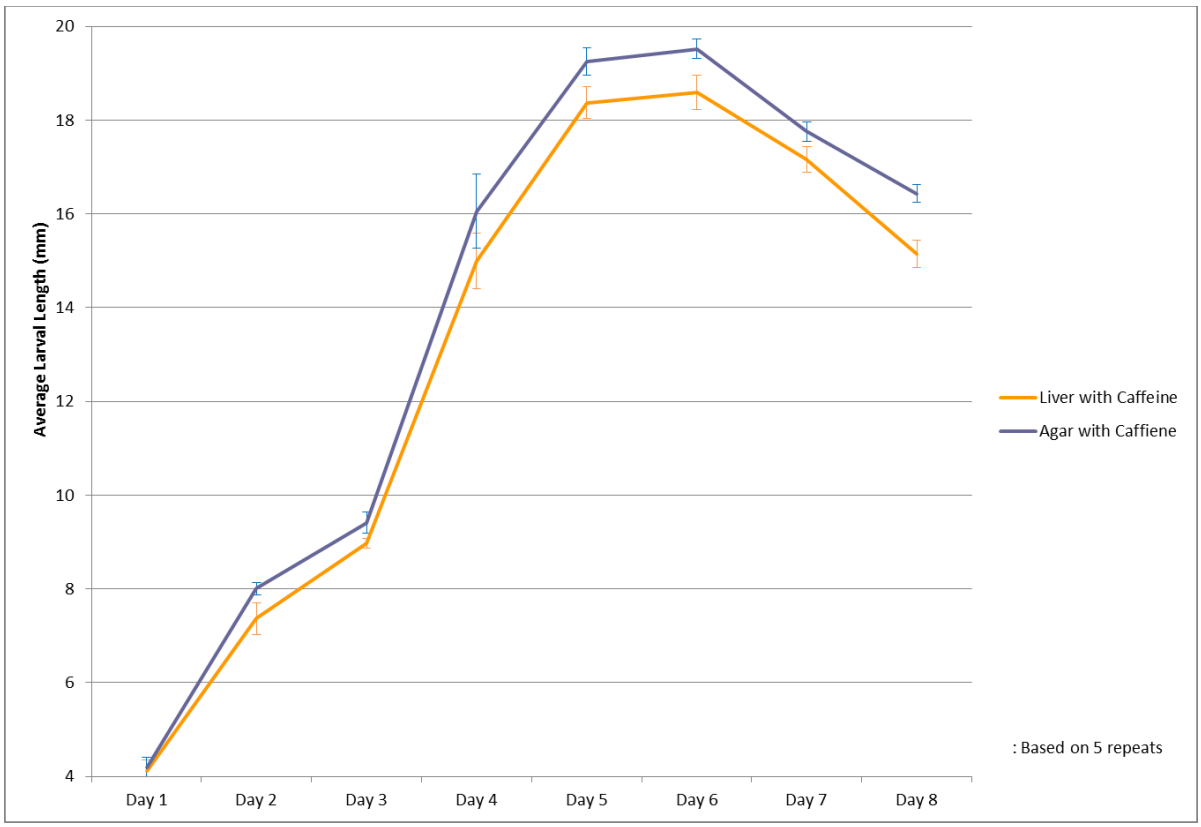


Figure 74: A comparison of average larval length across two diets.

4.9.3.2 Conclusion

It was concluded that the artificial diet with drug addition provided a more reproducible and standardized method for testing the effect of the drug variable when compared with minced liver with drug addition. The reasoning behind these differences is unconfirmed but the determined artificial diet does perform better.

4.9.4 Variance in larval food uptake in presence of drugs

To monitor the effect of Novel Psychoactive substances, first it must be determined that the presence of these drugs does not deter blowfly larvae from ingesting the food source.

The presence of drugs and the effect that they have on the decomposition of a cadaver is not known, except for the changes ensuing from the differences in behaviour observed when larvae intake drugs. An experimental plan to examine the effect of drug presence on decomposition was considered, however; determining decomposition rate is only relatable when analysing a full cadaver.

In order to investigate this, three separate diets were produced, two containing drugs (Paracetamol and Caffeine) and one control. A small number of larvae were placed at first instar onto these diets and behaviour (wandering/eating) was monitored. The food source was weighed daily to determine loss and larval development on each individual diet was monitored for differences. The diet weight was obtained by firstly removing the small number of larvae; it was assumed that water loss would stay approximately equal. The experiment on the three diets was replicated for consistency.

4.9.4.1 Results of variance in larval food uptake with drugs present

Shown in Figure 75 is a line graph displaying the average amount of diet consumed at each time point across three diets. If the diet is not seen as palatable then it was suspected that larval wandering would increase, as an alternative food source is located. Wandering was easily observed, as larvae would attempt to escape from the boxes through the holes in the lid. A measurement was also taken each day to determine how much weight had been lost from the diet. Agar diets are favoured due to their lack of

water loss although this must be considered during analysis of this data. Diet weight appears to decrease steadily across the 9 days during larval activity; the last sampling days appear to plateau as post-feeding stage is entered.

Excessive wandering was not observed across any of the diets. No particular difference is shown between the amount of diet lost, and larval mass does not appear significantly impeded. Larvae feeding on the caffeine diet actually appear slightly larger than the other populations; this could be due to the effect on caffeine on development.

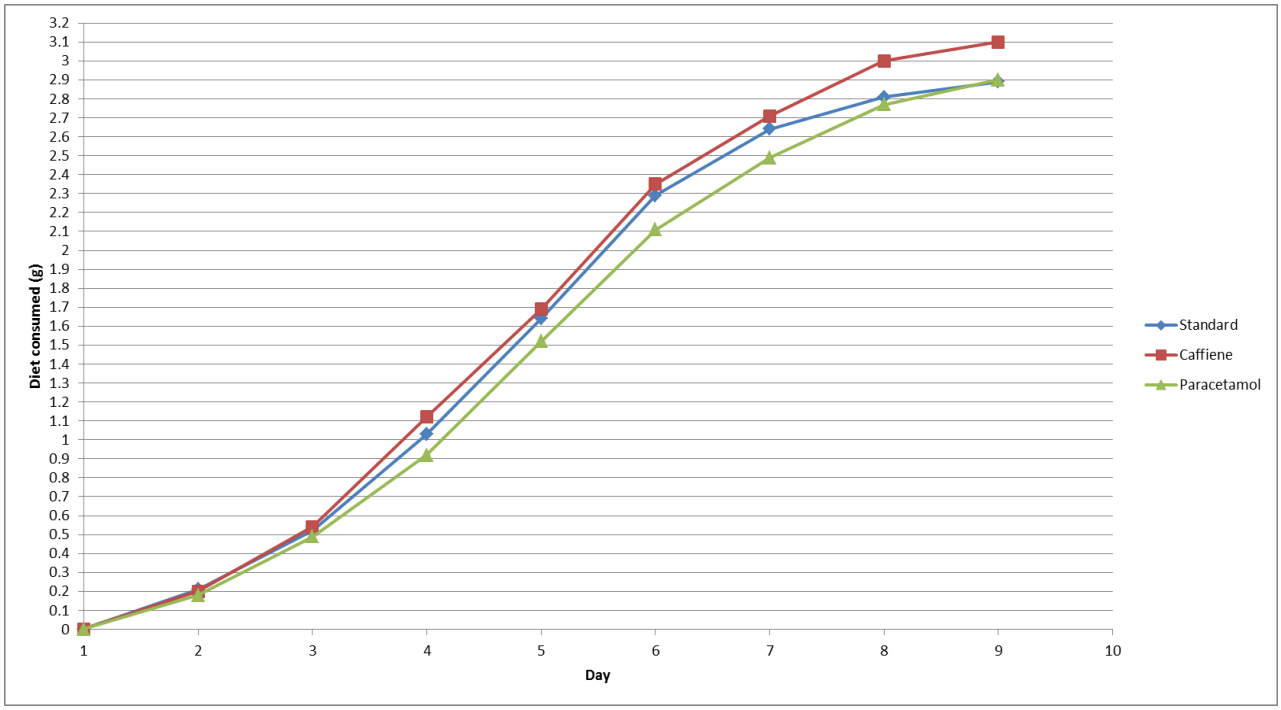


Figure 75: The variance observed in uptake of food in the presence of drugs.

4.9.4.2 Conclusion

Palatability is harder to control in artificial diet experiments where drugs are the main focus, however; this research shows that the addition of drugs in the similar quantities did not appear to affect larval behaviour, increase wandering or impede development due to decreased ingestion.

4.9.5 Variance observed during egg laying in the presence of drugs

It is of forensic interest to determine the effect, if any, on blowfly oviposition on a corpse where drugs are present. It is possible that drug presence will deter gravid blowfly females from laying their eggs.

Diets were therefore produced by mincing liver and then mixing either a high or a low dosage of three different drugs (Table 7). This was then presented to a blowfly population, allowed 10 hours to ensure maximum egg laying opportunity and then the numbers of eggs present were counted. The minced liver diet was selected here to ensure the effect on egg laying had resulted from drug presence not the diet itself. Each diet was replicated once to encourage reduction of the method limitations. This study is of limited use due to variables that were unable to be controlled. A specific colony of blowfly was kept for this experiment to ensure that the rearing colonies were not subject to any drugs. The colony was replenished for every drug separately tested and conditions kept as consistent as possible, because of this, these studies were undertaken over a long period of time, which will introduce its own variables. Blowfly numbers were kept approximately the same, but this cannot be guaranteed. It is also possible that the blowfly colony will have contained different numbers of males and females and therefore data is presented as an observation only.

4.9.5.1 Results of variance observed during egg laying in the presence of drugs

The average number of eggs laid on the control diet by the blowfly population was the highest number observed. All other diets with drugs present resulted in a slightly lower number of eggs when counted. The data shows that the higher dosage of all three drugs shown appears to display the biggest reduction in numbers of eggs. It appears from this dataset that drug presence does affect oviposition and high doses are affected more but this difference does not appear to be substantial. It is within the nature of the blowflies to reproduce and they do not know when they will next have a chance to lay their eggs and ensure the next generation, this likely eventually overpowers the effect of drug presence.

Table 7: Doses used for preliminary experimentation

Substance tested	High Concentration	Low Concentration
Caffeine	150 mg/kg	80 mg/kg
Paracetamol	1000 mg/kg	500 mg/kg
Blow	40 mg/kg	10 mg/kg

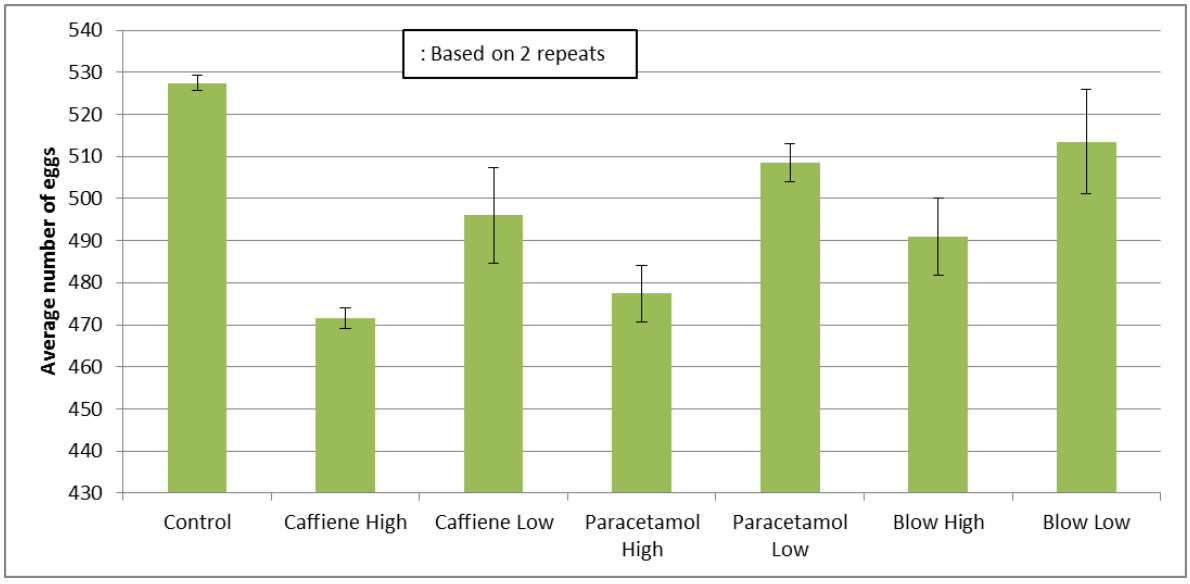


Figure 76: The average number of eggs laid for each drug within a 10-hour time period.

4.9.5.2 Conclusion

An observation made during experimentation was the presence of single eggs as well as clusters on the high concentration diets only. This has been shown in previous research [39], [291] to be the result of stress. Conditions were unlikely to have changed dramatically between the control, lower concentrations and the higher concentration diets so it is likely that this 'stress' was due to the high drug concentration presence.

4.10 Overall Conclusion

This chapter contains a mix of data, both essential for this thesis but also of interest for future research for other entomologists.

Firstly, the weight for an amount of eggs was determined to allow the transfer of accurate egg masses without causing damage resulting in mortality.

It was necessary for the use of pre-existing samples to determine the effects of killing methods on the cuticular hydrocarbons. It was shown that neither killing with hot water or deep freezing has a significant impact on the cuticular hydrocarbon profile, a slightly lower concentration was observed, however this did not affect analysis.

Preliminary testing of cuticular hydrocarbon differences over time after the death of a blowfly showed some chromatographic differences for different time groups. This experiment was inconclusive however, due to the variations shown initially, owed to differing age upon death; this is nonetheless realistic.

Protocols were also refined for cuticular hydrocarbon extraction and solvent selection and an optimised derivatisation method for the analysis of NPS was defined.

An artificial diet was developed for use as a method to enable homogenous delivery of specific drugs to blowfly larvae for determination of developmental differences. Testing of diet preferences when a drug was added showed no problems for future diet use.

Egg laying in the presence of drugged diets was monitored and differences shown. Single egg laying was observed along with a slightly lower number of eggs on drug diets.

5.0 Introduction

The effect of the toxins or drugs on the growth and development of the blowfly is of great research interest, as it can affect PMI estimations [91],[93]. Different species appear to have different responses to drugs and the rate in which they develop can increase, decrease or stay the same [148]. The reaction of the species to the presence of a drug may not always mirror that experienced by the human user, for example; stimulants may not increase activity, just as depressants may not reduce it. A number of papers have shown that drugs have an effect on growth rates and due to the role of these measurements within the field of entomology, can lead to inaccurate PMI estimations. It is important, given this information that all factors with a potential influence are researched. Additional research has shown drugs and toxins interfere with usual larval development and consequently require extended research to determine correct developmental data considering these conditions [140], [151], [152]. Sometimes just the presence of the toxin will affect the insect but in other circumstances it will need to be present in a certain concentration before having any influence upon development [99].

This experiment was carried out to determine if the presence of a drug, in this case a range of Novel Psychoactive Substances (NPS) and common adulterants, affects traits used within the field of Forensic Entomology, to age larvae, predominantly larval length and weight. The lack of research focussed on Novel Psychoactive substance effect on

forensically important blowfly and the extraction of these compounds suggested this focus.

Larvae were reared in equal groups on pre-researched artificial diets (the reader is referred to chapter 4) containing a specific drug; instar, weight and length was then measured each day from emergence. This data were then compared to other tested drugs and a control group to determine the effect of the variable.

Results from previous experimentation based on artificial diets showed growth rates comparable and often exceeding that of animal tissue. An artificial diet was decided upon for the ease of drug addition and the ability to enable even drug distribution. Studies performed prior to this experiment had also shown no significant difference in growth measurements comparing animal tissue with a drug addition and the chosen artificial diet with the drug addition. This enabled the utilisation of an artificial diet with a fair assumption that these variables were controlled. Further experimentation also showed no difference in uptake of the diet in the presence of drugs, meaning any growth differences were likely due to the effect of the drug not purely its presence.

5.0.1 Adulterants

Part of this investigation was focussed on common drug adulterants. Adulteration, bulking and contamination of substance of abuse is common place. Many components have been found, such as benign substances like flour and sugars, substances to enable administration of a drug, such as caffeine to allow smoking of heroin or cocaine and finally substances that mimic or enhance the effect of the drug. Negligence during the

manufacturing process can also result in contamination with components such as lead [292].

Three of these components were selected based upon knowledge of frequent adulteration substances. Caffeine, which is a psychoactive stimulant drug in its own right, is very popular for use in this manner. As a stimulant, it can be added to other stimulants such as amphetamines to create a similar effect at a cheaper price. It is also used for addition to heroin for smoking as it allows vaporisation at a lower temperature [292]. Benzocaine is a local anaesthetic, which is popular for cutting with cocaine due to the similar effects produced. It can also be added to drugs to enable administration by smoking or to relieve pain from injection [292]. Acetaminophen or Paracetamol, as it is more commonly known, is again a cheap alternative, which is readily available. It is known for its pain relief. A popular drug to be cut with paracetamol is heroin, due to the analgesic (pain killing) effects but also as it is used to disguise poor quality heroin using its bitter taste [292]. It was important to include these within the study due to the frequency that these substances are found in not only NPS but also other illicit substances and the concentrations often observed. These substances can cause considerable harm on their own also, in larger doses and in certain circumstances, death. Studies have been shown to purify the drug of interest to remove adulterants which provides an unrealistic situation [150]. One study showed that users of NPS were putting themselves at risk of significant caffeine toxicity due to the unexpected, but high caffeine concentrations of caffeine determined during analysis [178]. These compounds must be considered due to their presence in NPS and other drugs but also due to their individual toxicity, which may become forensically relevant.

5.0.2 Aims and objectives

The aim of this experiment was to determine the effect of 11 chosen Novel Psychoactive Substances, 3 common adulterants and drugs within their own right and 1 illegal drug to be used as a comparison, on the development of blowfly larvae. It was hypothesized that differences would be seen at different stages for different drugs due to the range of published research (reader is referred to Chapter 1.2.3), so measurements were taken daily until 50 percent of the sample group had pupariated. If the addition of these components to the larval diet produced developmental differences, then this could potentially lead to incorrect PMI estimation.

The collection of data is described in the following section including the use of statistical methods for analysis. Results are shown in the next section with a variety of boxplots demonstrating larval length and weight at different time points and PCA plots describing the observed differences and showing any clustering of the data. Findings are discussed amongst results due to the larger amount of data shown. Conclusions close this chapter and summarize before the reader continues on to the analysis of cuticular hydrocarbons in Chapter 6. Drug analysis will be discussed in Chapter 7.

5.1 Materials and methods

Colony sources are outlined in Chapter 3.1. Meat was introduced to a colony of *C.vicina* initially and then a colony of *L.sericata* to initiate and enable egg laying, a strong colony was necessary to ensure a large number of resulting eggs. Blowfly eggs were monitored carefully to determine start of hatching. Before the majority had hatched, the mass

equivalent of 600 eggs was taken using soft tweezers or a paintbrush so not to cause damage and then placed onto the diet prepared as set out in Chapter 3, with a predetermined drug addition. A mass of 600 was chosen to enable sufficient numbers for collection and harvesting of daily samples, allowing for a percentage of mortality and a small number remaining after pupariation to use for further experimentation into the effects of drugs on emergence from pupae, health of resulting adults and ability to lay viable eggs. 600 larvae was chosen also to account for and control the possibility of overcrowding, competition, maggot mass effect and any developmental changes associated with these variables. This should result in data where developmental changes are due to the drug.

To produce the diets, the fresh liver tissue was taken and blended until a meat paste was formed. 90 g of this meat paste was then added to a large petri dish for each diet. In a separate beaker, 9 g of agar powder and 187.5 ml of distilled water were added together and mixed. This mixture was microwaved in one-minute bursts until the solution was completely clear and all agar powder had dissolved. This was then cooled to 60 °C before adding the calculated amount of drug (Table 8). This mixture was then quickly added to the blended meat and mixed thoroughly with 22.5 ml of blood, then left to set. All petri dishes were clearly labelled with drug and concentration. Two diets were prepared for each population, allowing 1 g of diet per larvae. Once these larval masses were placed upon the diets, they were put into purpose bought containers with sawdust lining to ensure a more preferable humidity level; placed in an incubator with a constant controlled temperature of 23°C.

Larvae were not disturbed at this stage for 12 hours to ensure they settled in to feeding. After this at each 12-hour period, the larval populations were removed from the incubator.

Ten larvae were then selected at random to firstly be weighed using an Ohaus analytical balance, accurate to 0.0001 g. Further larvae were then collected according to the sampling day (see chapter 3). A larger number of larvae were required during initial instars due to size and the need to use collected samples for further experimentation of cuticular hydrocarbons. Larvae were killed by submerging them in water just below its boiling point, for around 30 seconds. Instar was identified as shown in chapter 3 and larval length was measured with Digital Vernier Calipers, accurate to 0.01 mm to enable calculation of an average.

This experiment was carried out for 11 NPS, 3 common adulterants, 1 illegal comparison and a control population. Two dosages were investigated per drug and each was repeated to improve accuracy of results. Dosages were chosen based upon post mortem concentrations shown in literature following an overdose. When this information was not available, as the substances had not been heavily researched, estimation was made based upon known dosage amounts or post mortem concentrations of substances that are chemically similar [109], [113], [169], [178], [293]–[305], (Table 8).

Experimentation was also carried out using two drugs and a control population on *L.sericata* to observe species differences. Again, samples were taken daily until 50 percent of the sample population had pupariated; this time differed for each drug diet.

Once pupariated, pupae were carefully transferred to plastic cups with dry wood shavings until emergence from the puparial case. Upon emergence, adults were released into smaller rearing containers where behaviour was monitored. Milk powder, sugar, water and liver were provided as normal. After giving a period of time to allow maturity of the blowfly ovaries, animal tissue was provided to determine the ability of these blowfly populations reared in the presence of drugs, to lay eggs.

Table 8: Dosages used in experimental study.

Substance tested	High Concentration	Low Concentration
AMT	40 mg/kg	25 mg/kg
6APB	92 mg/kg	25 mg/kg
6+5APB	92 mg/kg	25 mg/kg
5EAPB	92 mg/kg	25 mg/kg
Benzofury Blue	92 mg/kg	25 mg/kg
Benzofury Beige	92 mg/kg	25 mg/kg
Benzofury Green	92 mg/kg	25 mg/kg
MDA	92 mg/kg	25 mg/kg
Benzocaine	1200 mg/kg	750 mg/kg
Ivory Wave	72 mg/kg	45 mg/kg
Caffeine	150 mg/kg	80 mg/kg
Paracetamol	1000 mg/kg	500 mg/kg
Pink Panther	20 mg/kg	9 mg/kg
Synthacaine	70 mg/kg	36 mg/kg
Blow	40 mg/kg	10 mg/kg

All compounds were kept according to guidance of the home office and under the direction of the schedule one license kept onsite. During the period of experimentation, compounds were habitually reclassified and required specific storage and usage guidelines to be followed.

5.1.1 Statistical Analysis

Data collected was analysed using two statistical packages, R (version 3.4.0) and Unscrambler (version 10.5).

Firstly, length and weight data were accumulated in an excel spreadsheet and minimum, maximum, mean and standard deviations were calculated for each drug at each time point. 2D line graphs were produced using mean measurements to provide an overview of selected diets in comparison with a control across the developmental period. Scatter plots were also produced to illustrate the relationship between the two variables.

The two different dosages were combined for initial analysis; the effect of the dose was investigated later on; the reader is directed Chapter 5.2.4.

Data were then imported into code driven statistical software 'R', QQ norm plots were produced for each data set to ensure that the data was normally distributed, an example is shown in Figure 77. The linearity of the points suggests that the data set is normally distributed. A residual vs fitted plot was also created to show if data was of uniform variance. All data sets were normally distributed and had uniform variance, an example is shown in Figure 78.

The next stage used a mixed effects model where the larval length and weight were compared individually for all 16 diets. This determined if the developmental data across the different diets was statistically different. This difference was then inspected further using Tukey tests. This is a pairwise comparison test and focuses on where the differences were, and which were most significant. Boxplots were also produced, the bottom and top of the box show the first and third quartiles of the data, and the band inside the box is the median.

Data were then input into 'Unscrambler' where a weighted PCA was produced. PCA shows both length and weight data together. Data were weighted to allow a common scale. This enabled visualisation of data clustering across the different time points. Principle components were selected based upon their explained variance.

Hoteling's T^2 statistic is calculated based on the samples used when calibrating the model, those exceeding the limits set are deemed outliers, and this means that the samples are well explained by the model but represent variation. The limit set for analysis within this research was 5%.

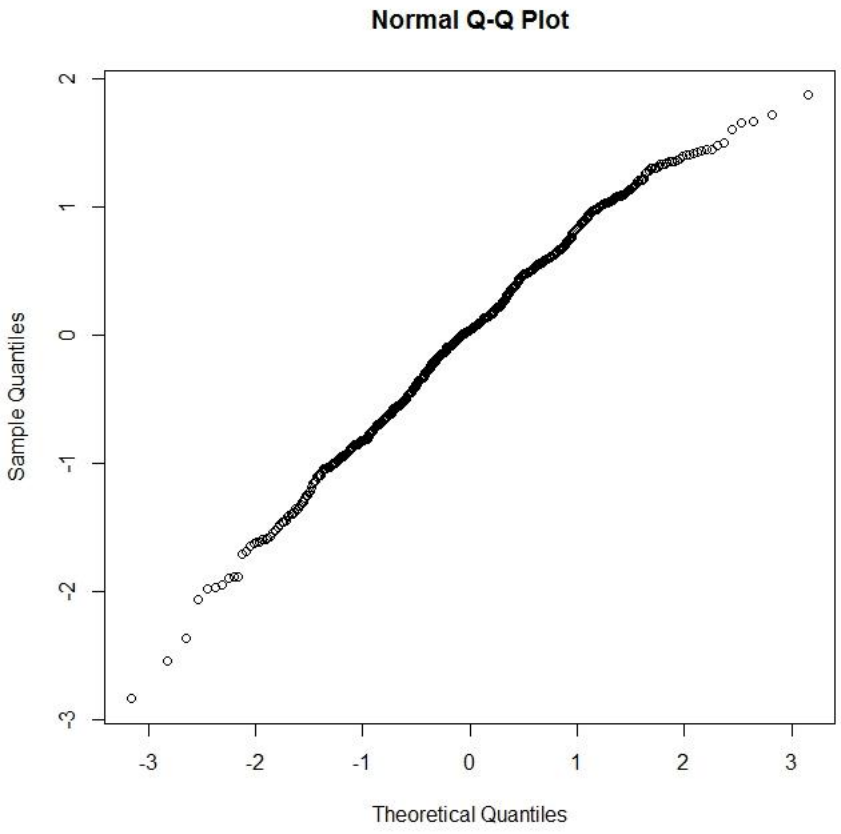


Figure 77: Example QQ Norm plot showing normality of data.

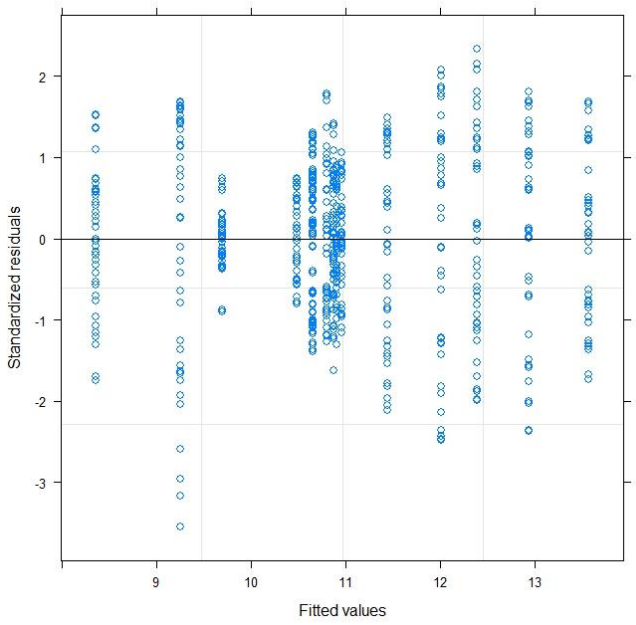


Figure 78: Example of Residual vs fitted plot to show data variance.

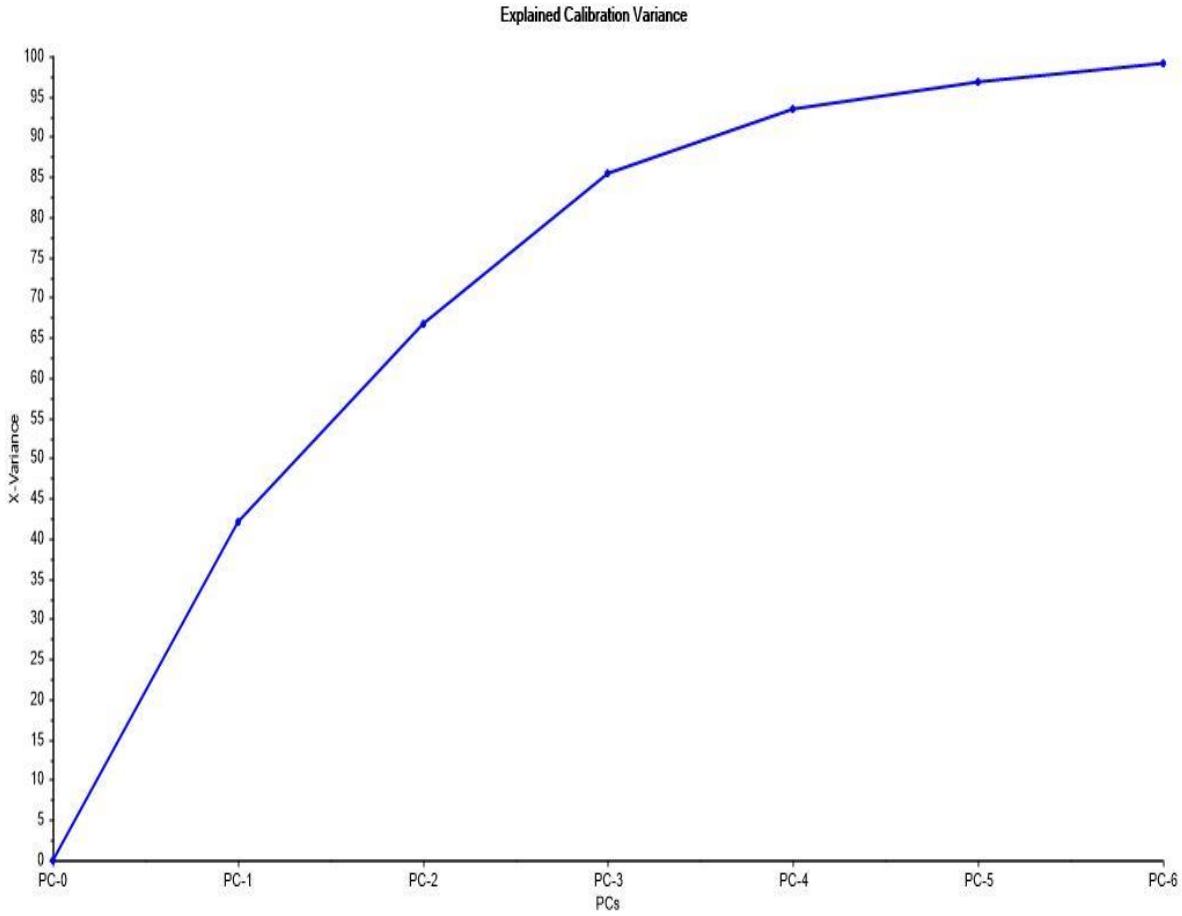


Figure 79: Explained variance plot associated with PCA.

Explained variance plots were produced in Unscrambler and were used to select appropriate principle components for displaying sample variance, an example is show in Figure 79. Each principle component explains a percentage of the population variance, in order to represent the variance observed, principle components must be carefully selected. Principle component 1 explains the largest amount of the population variance and this information decreases as the PC numbers increase. It would be misrepresentation of a dataset to use only PC representative of a low percentage of the total variance. These plots are often shown to plateau and at this point, no further information regarding population variance can be achieved.

5.2 Results and Discussion

5.2.1 Combining data repeats

Before combining the data from both repeats together for analysis, it was analysed to ensure that there was no significant difference between the two data sets. PCA was used to display control length and weight data for alternate sampling days for both repeats separately. This can be seen in Figure 80 and sampling days do not show separation due to the combination of two datasets. All datasets were therefore combined for all analysis.

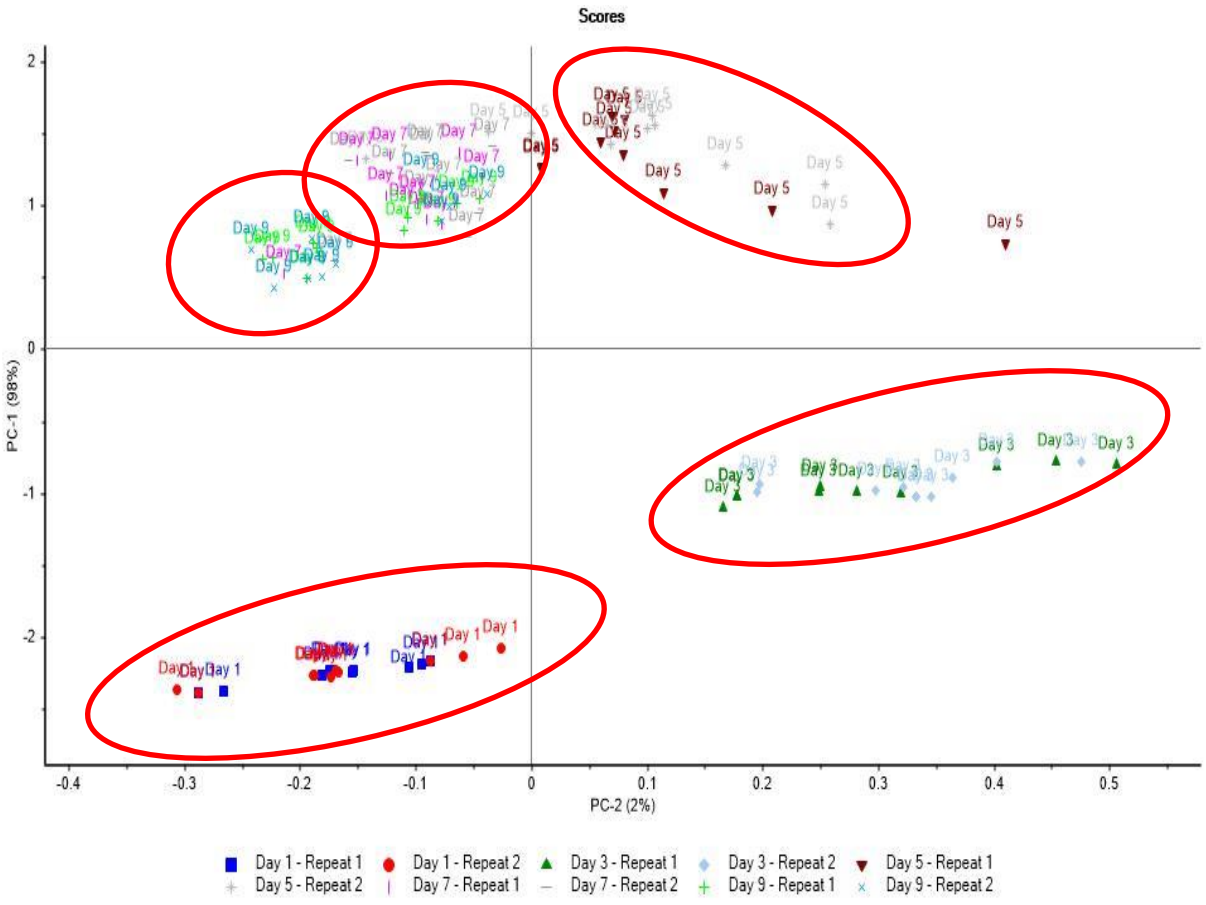


Figure 80: PCA plot showing no difference between repeats of the control population (n=20).

5.2.2 Pupariation time and instar

Instar was recorded every 12 hours throughout development in order that an estimation of developmental progression could be determined. This data are shown in a bar chart in Figure 81 and Figure 82. The higher dosage of paracetamol appears to delay progression through the life stages and ultimately time taken to reach pupariation. This dosage increased the larval stages by 48 hours when comparing with the control population. Second instar duration appears to double in comparison with all other sample groups; third instar is also increased by 24 hours, as is the post feeding stage. The lower paracetamol dosage however did not impede development, pupating 24 hours prior to the control population. All other treatments pupariated at least 24 hours, if not 48 hours in some cases, before the control population. The stages of development stayed the same for all treatments (except for high dose paracetamol) during 1^{st}, 2^{nd} and 3^{rd} instar with the differences being seen during the post feeding stage. In relation to instar, discounting paracetamol, all other drug treatments seemed to show no difference when comparing drug dosages. Comparison of 6APB and MDA, the drug believed to be chemically similar also shows no difference in relation to instar duration or pupariation time.

It is noteworthy that all changes in instar were observed at 24-hour periods.

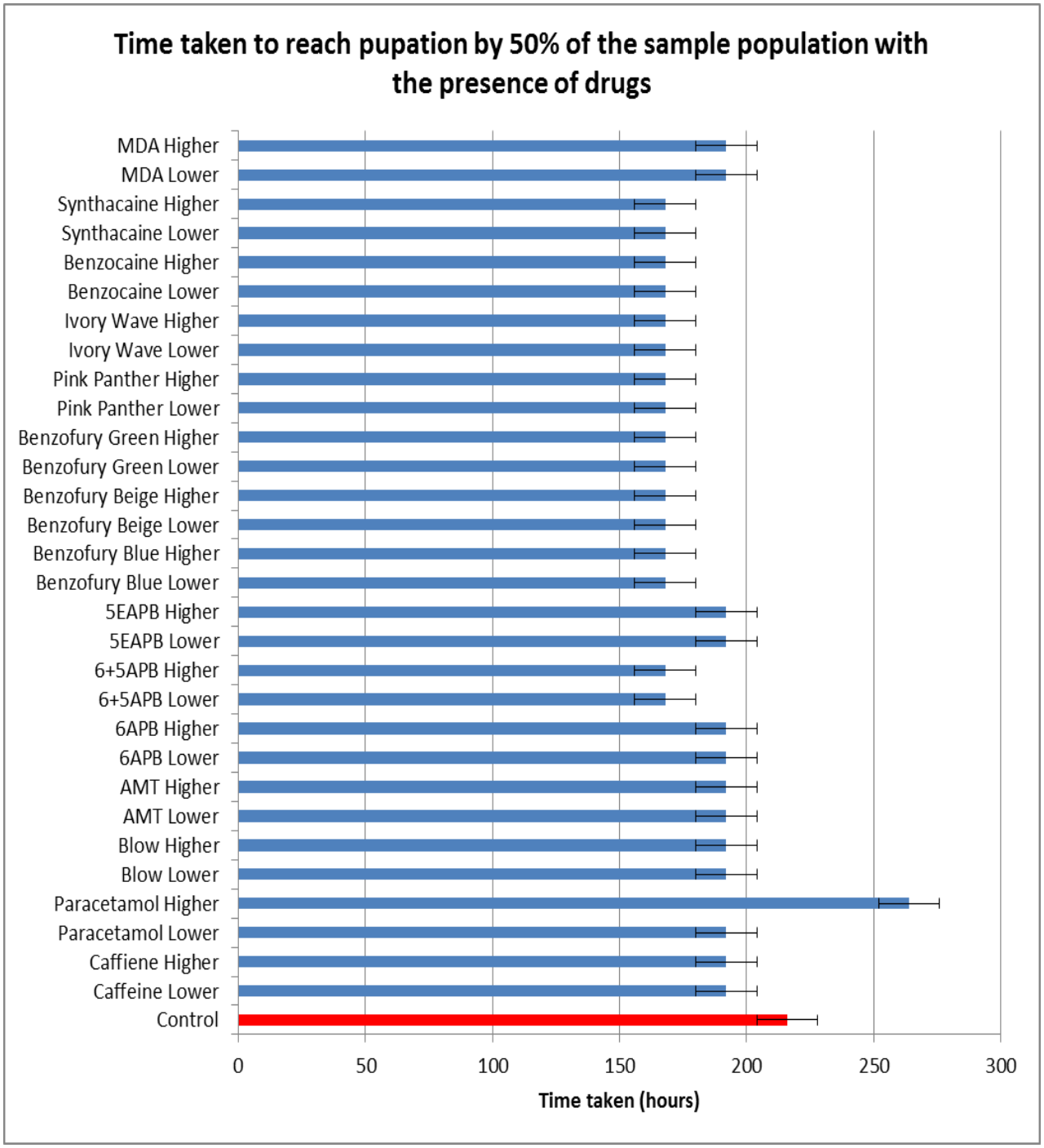


Figure 81: The time taken for 50% of the population to reach pupariation for each drug diet, including error bars for C.*vicina*.

Figure 82: The duration of each instar during the developmental time of blowfly larvae in the presence of NPS for C.*vicina*.

An overview of a selected few drugs are shown across the whole duration of development in Figure 83 and Figure 84. This was then broken down to look at variances at each time point.

From Figure 83, larval length is shown to change dependant on the drug present from 24 hours after initially being placed on the diet. The steeper the line, the faster the development between those time points. Development of 'Synthacaine' is shown to be faster between day 1 and day 5 after which it is likely post feeding occurs due to the shrinkage observed, followed by earlier than expected pupariation. Paracetamol and AMT show a slower development from day 1 to day 3; at this point growth accelerates for the population feeding on AMT, where paracetamol is still much slower. The population consuming AMT reach a larger larval length, significantly larger than the control population and pupate at day 8, prior to the control at day 9, paracetamol does not pupate until day 11.

An overview of larval weight mean measurements show a very similar pattern as previously described (Figure 84). Weight increases with development until the arrival of post feeding where weight tends to level out as larvae move away from the food source in search of a suitable pupariation site, as explained in chapter 1.

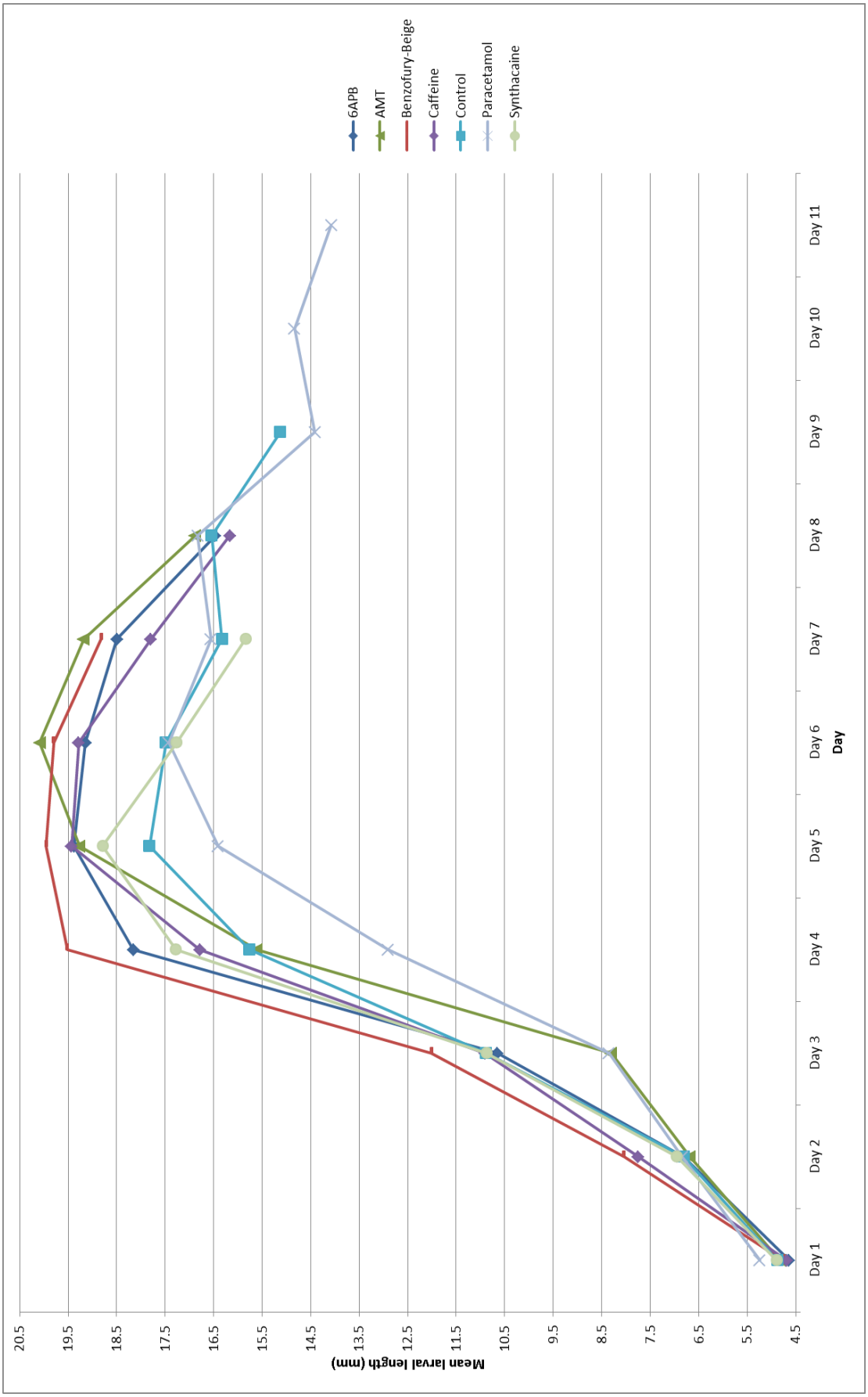


Figure 83: The mean larval length (n=40) from selected drug diets across duration of development.

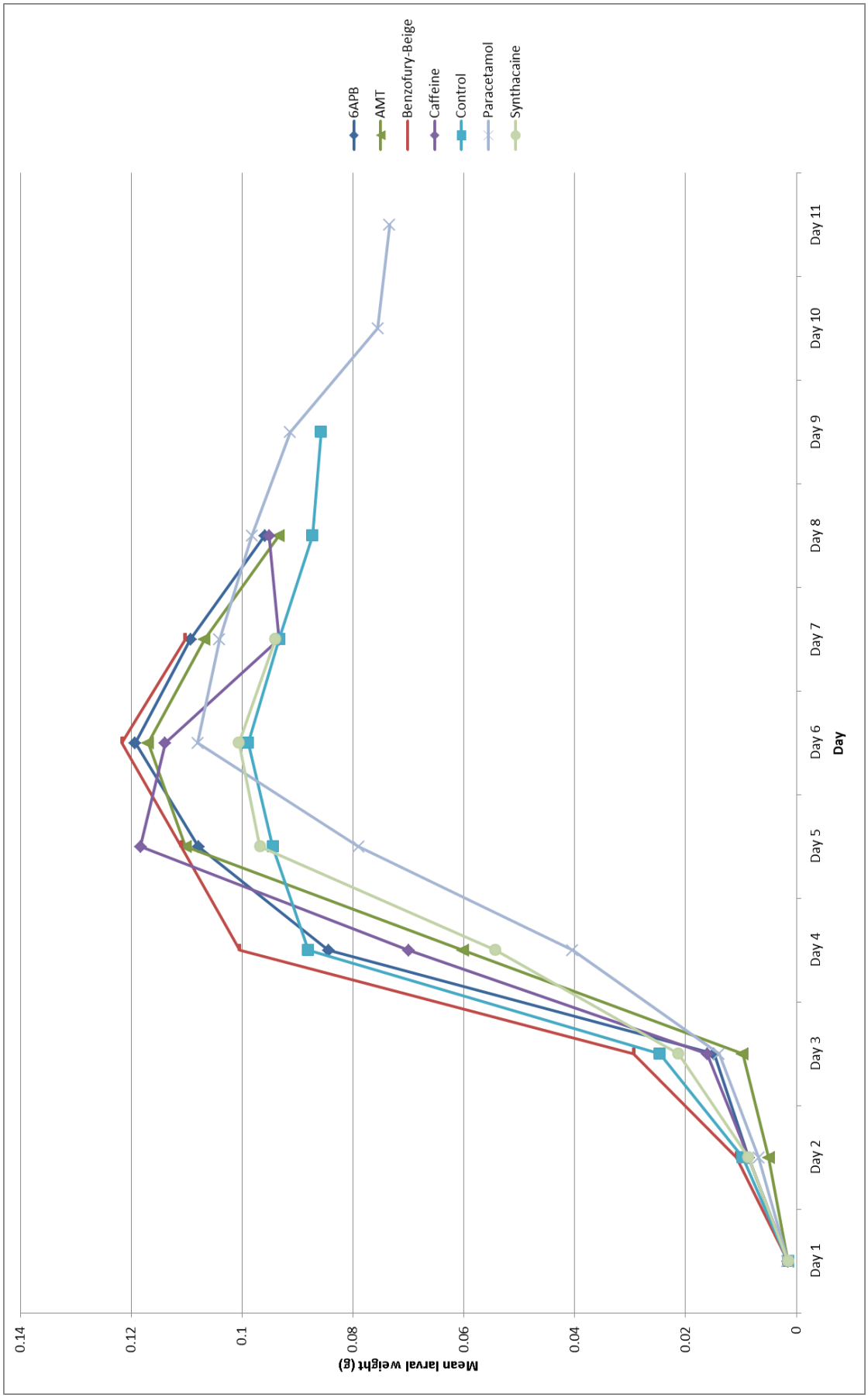


Figure 84: The mean larval weight of (n=40) from selected drug diets across duration of development.

5.2.3 The effect of NPS on the development of the blowfly, compared to the control population.

Day 1- Results

Initial observations regarding larval length at this time point did not appear to vary substantially. No larvae stood out as particularly larger or smaller than those of other sample populations. Larval lengths ranged from 3.05mm to 6.92mm (Table 9), showing a natural and expected variation.

When tested using a mixed effects model comparing lengths across the different diets, statistically significant differences were present (F=2.4397, P=0.0018). Data set was normally distributed and uniform in variance.

Larval Length

Further analysis (Figure 85) showed the majority of the data at day 1 to not be significantly different, with one exception. Larvae feeding on 5EAPB were significantly shorter (mean=4.4806mm, SD=0.7727mm) than those feeding on Paracetamol (mean=5.2395mm, SD=0.9988mm), ($p<0.01$), Pink Panther (mean=5.2125mm, SD=0.9335), (p=0.0119) and Benzofury Blue (mean=5.1528mm, SD=0.9764), (p=0.0354). However this is not significantly different from the control population (mean=4.8825mm, SD=0.5371) so it is not as important within this investigation as the focus remains on comparison with non-drugged larvae. A number of outliers can be seen within the population feeding on 5EAPB so it can be speculated that there are similar range of uneven data resulting in a lower mean.

Larval Weight

As observed with larval length, no obvious visual differences, other than natural variance, were observed across the test populations at day 1 (Figure 86). Larval weights ranged from 0.0005g to 0.0032g (Table 9). Testing with a mixed effects model returned no statistically different results (F=1.2597 , P=0.2227). A few outliers are observed within the population but it is hypothesized that this is due to an uneven spread of data as they are not beyond what would be expected. The observed range of data is approximately equal across all drug diets.

A scatter graph of all data from individual larval samples displays both weight and length and shows positive correlation between the two variables. This is expected because as larval length increases, as does larval weight, comparison was drawn at a later experimental time point after further exposure to the research drugs.

Principle component analysis was carried out using PC 1 and PC 2, together explaining 100% of sample variance, shown in Figure 88. Hoteling's T^2 statistic at a 5% significance level is included to provide information on potential outliers or those statistically very different. Data points are spread across the plot showing no pattern, no clustering is observed and numerous overlapping values are shown.

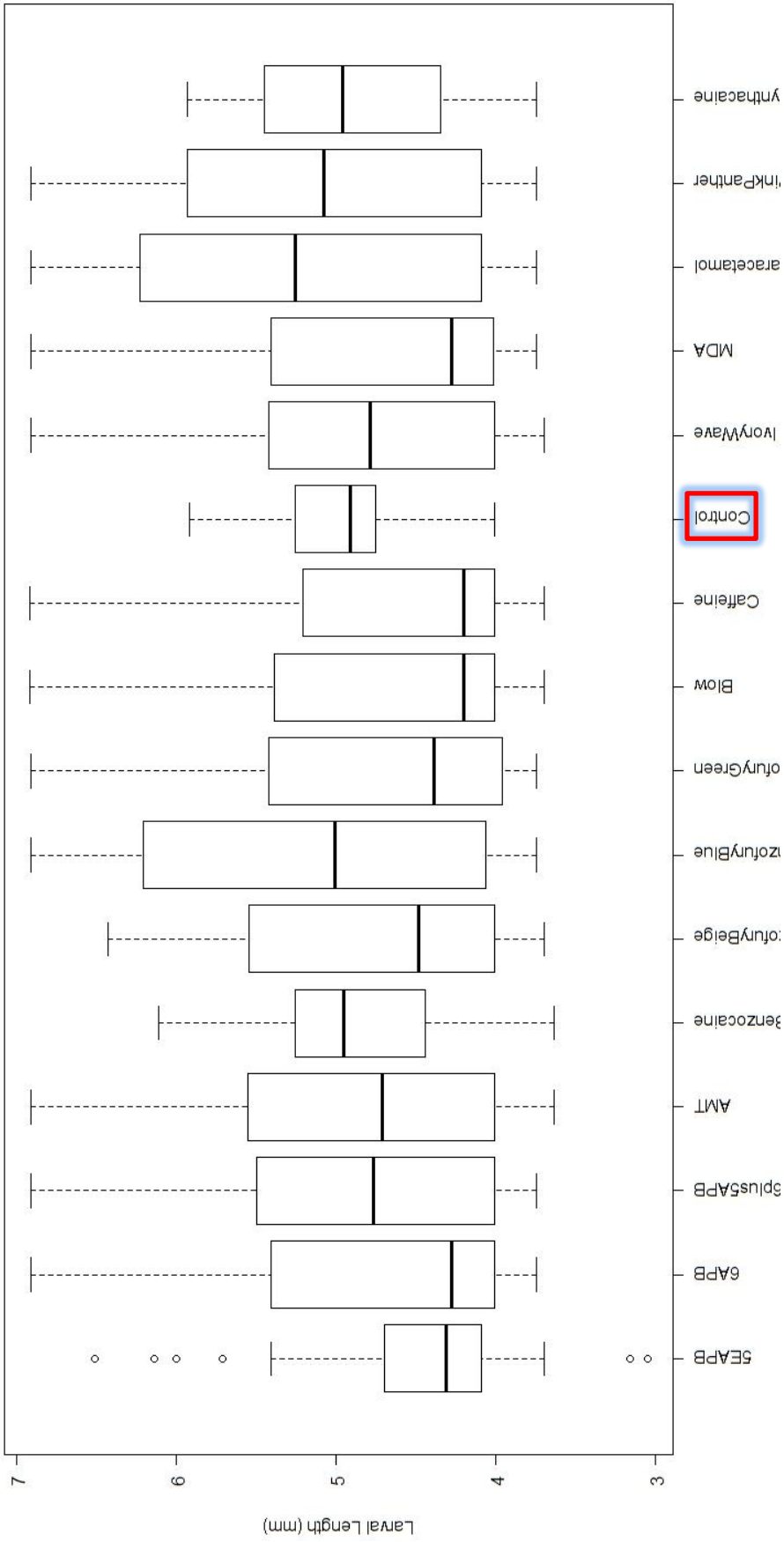


Figure 85: Box plot showing larval length for day 1 across all samples (n=40).

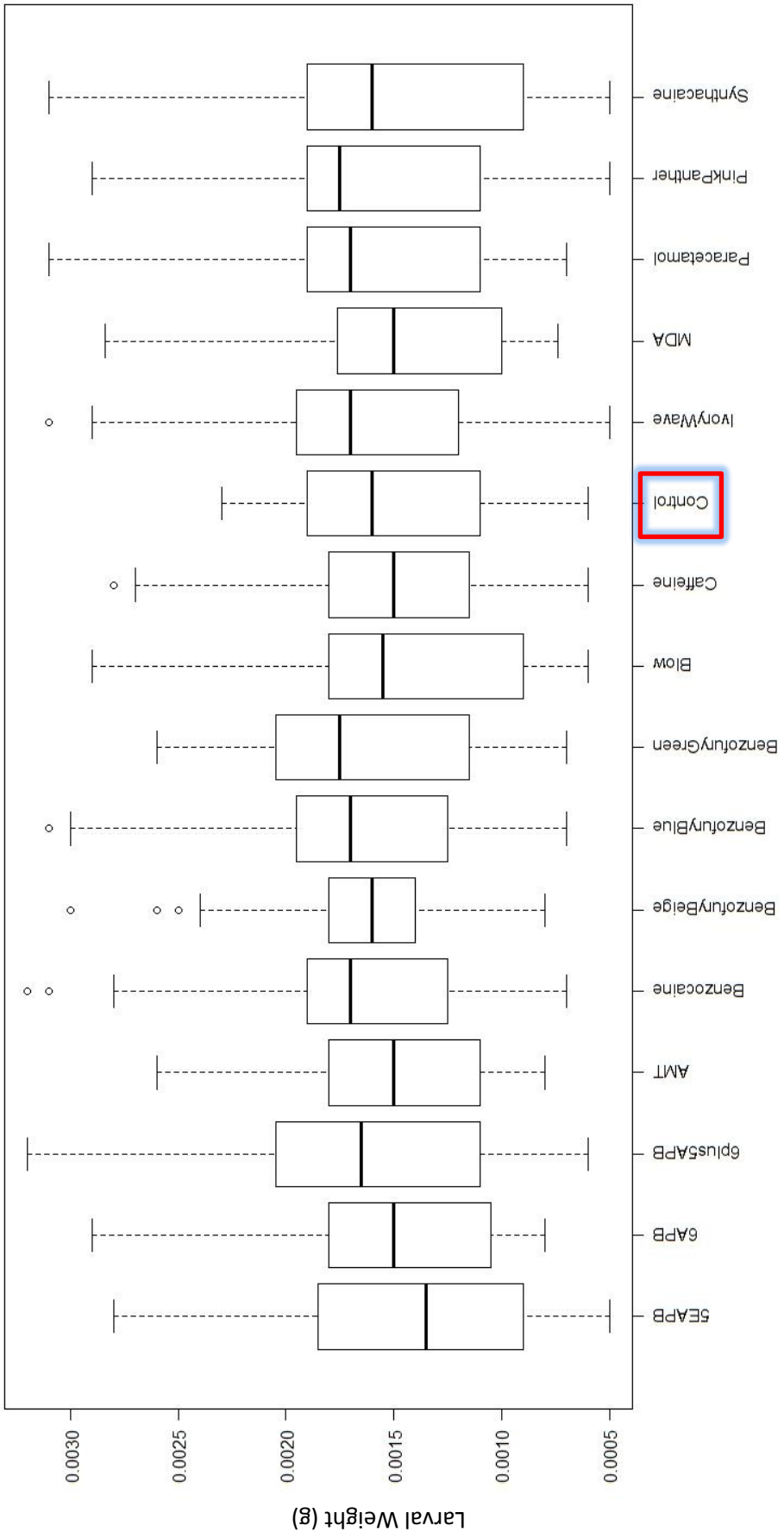


Figure 86: Box plot showing larval weight for day 1 across all samples (n=40).

Table 9: The minimum, maximum and mean lengths and weights (including standard deviation) at 24 hours (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	3.05	6.51	4.4806	0.773	17.25
	Weight (mg)	0.5	2.8	1.4	0.600	42.86
6APB	Length (mm)	3.75	6.91	4.6403	0.921	19.85
	Weight (mg)	0.8	2.9	1.5	0.500	33.33
6+5APB	Length (mm)	3.75	6.91	4.8158	0.903	18.76
	Weight (mg)	0.6	3.2	1.7	0.700	41.18
AMT	Length (mm)	3.64	6.91	4.823	0.937	19.44
	Weight (mg)	0.8	2.6	1.5	0.500	33.33
Benzocaine	Length (mm)	3.64	6.11	4.8565	0.621	12.79
	Weight (mg)	0.7	3.2	1.7	0.600	35.29
Benzofury-Blue	Length (mm)	3.75	6.91	5.1528	0.976	18.95
	Weight (mg)	0.7	3.1	1.7	0.600	35.29
Benzofury-Green	Length (mm)	3.75	6.91	4.7463	0.919	19.35
	Weight (mg)	0.7	2.6	1.7	0.500	29.41
Benzofury-Beige	Length (mm)	3.7	6.43	4.7653	0.855	17.94
	Weight (mg)	0.8	3	1.6	0.500	31.25
Blow	Length (mm)	3.7	6.92	4.6983	0.942	20.04
	Weight (mg)	0.6	2.9	1.5	0.600	40.00
Caffeine	Length (mm)	3.7	6.92	4.688	0.893	19.04
	Weight (mg)	0.6	2.8	1.5	0.500	33.33
Control	Length (mm)	4.01	5.92	4.8825	0.537	11.00
	Weight (mg)	0.6	2.3	1.5	0.500	33.33
Ivory Wave	Length (mm)	3.7	6.91	4.837	0.887	18.33
	Weight (mg)	0.5	3.1	1.7	0.600	35.29
Paracetamol	Length (mm)	3.75	6.91	5.2395	0.999	19.06
	Weight (mg)	0.7	3.1	1.6	0.600	37.50
Pink Panther	Length (mm)	3.75	6.91	5.2125	0.934	17.91
	Weight (mg)	0.5	2.9	1.6	0.500	31.25
Synthacaine	Length (mm)	3.75	5.93	4.892	0.622	12.71
	Weight (mg)	0.5	3.1	1.5	0.600	40.00
MDA	Length (mm)	3.746	6.914	4.6403	0.921	19.84
	Weight (mg)	0.7	2.8	1.5	0.500	33.33

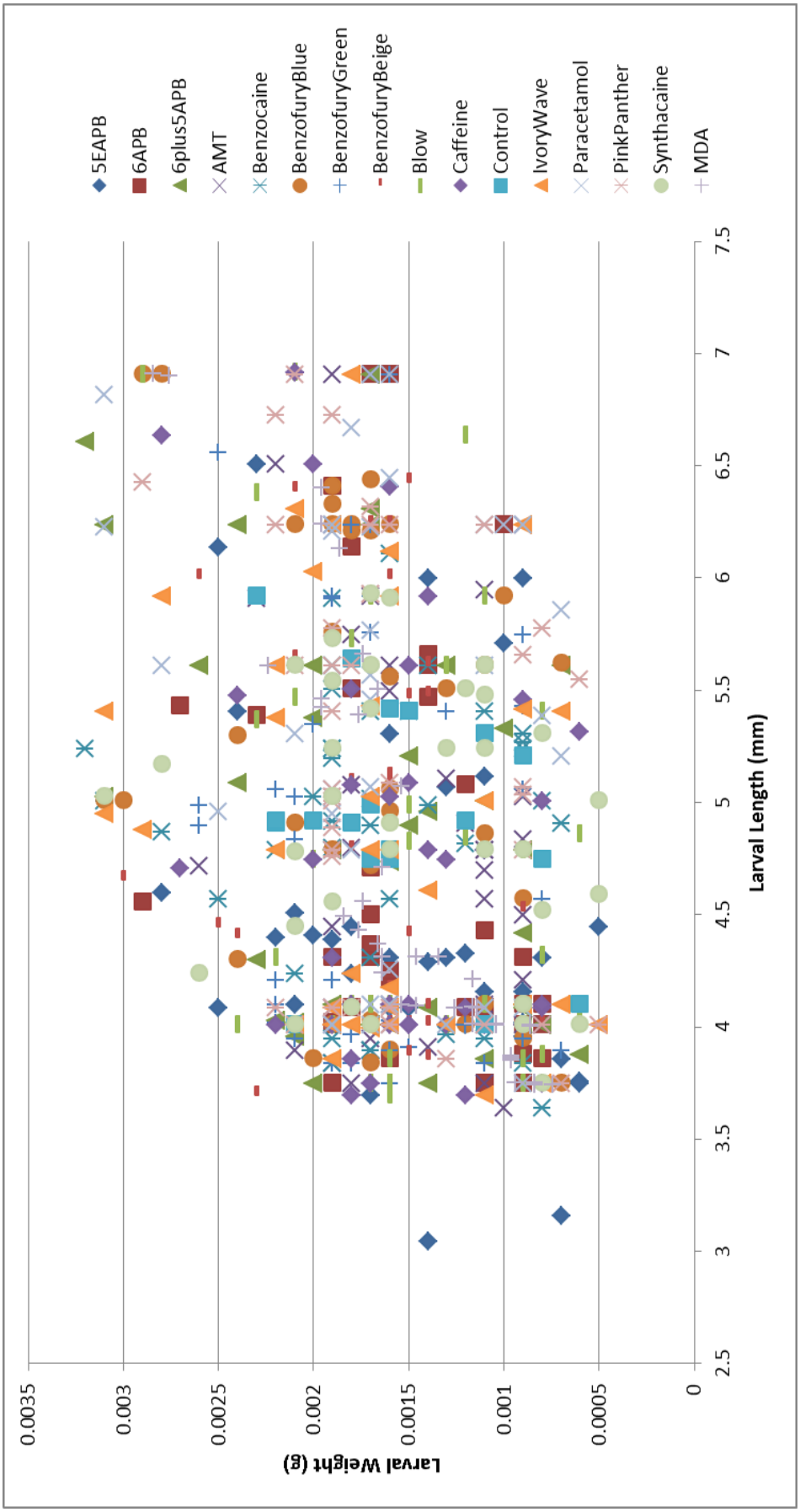


Figure 87: Scatter graph showing relationship between length and weight data observed at 24 hours.

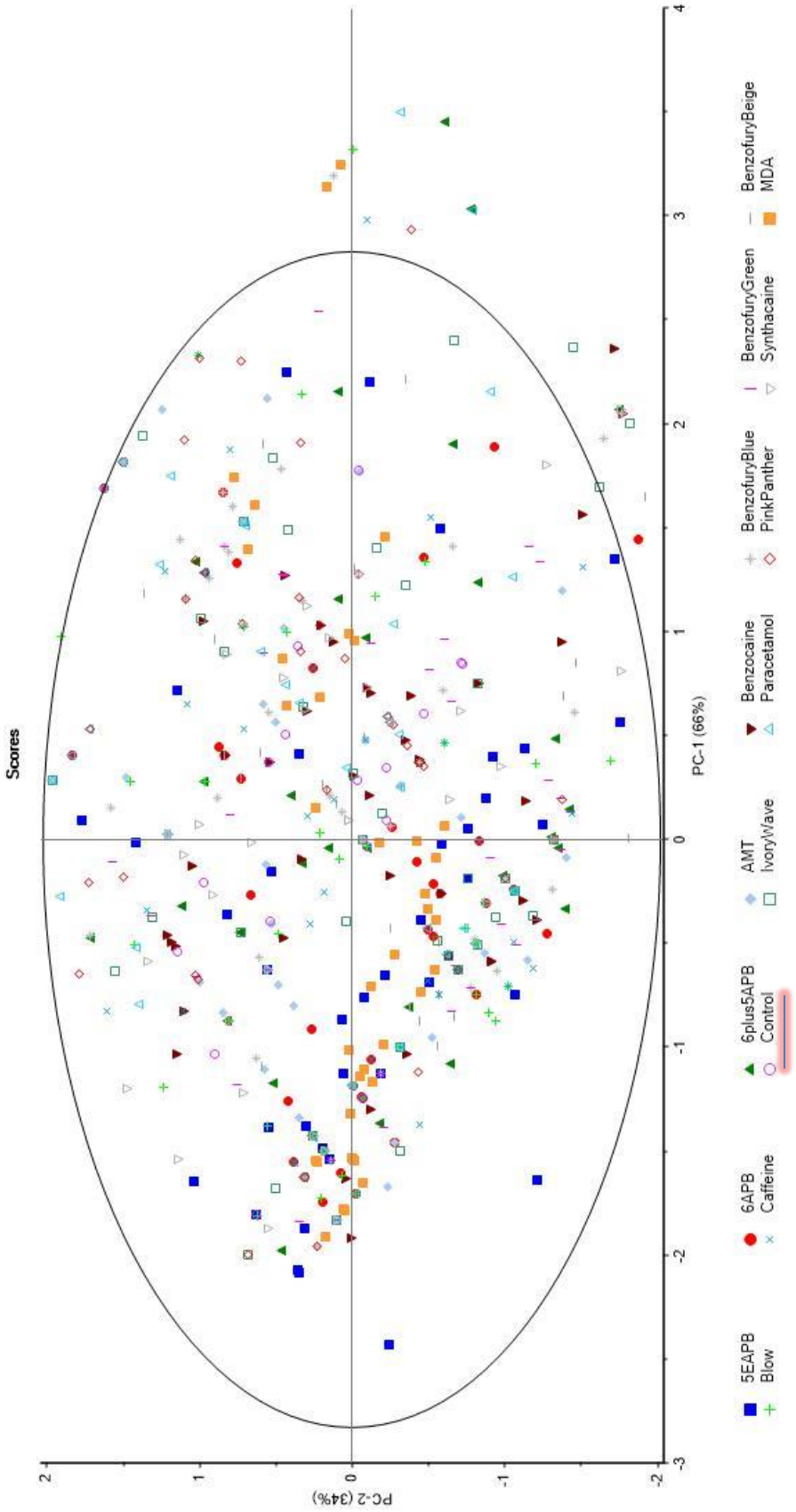


Figure 88: PCA plot showing all drug additions at 24 hours.

Day 1: Discussion

At 24 hours, most sample populations showed no significant differences for both larval length and weight. One exception to this was length of larvae from the experimental population given 5EAPB, which was shown to be significantly different to Paracetamol, Benzofury Blue, and Pink Panther diets but not to the control. It is hypothesized that this is due to an uneven spread of data across the sample population as the length range is in line with the range observed across other diets and a number of outliers are identified in the higher larval length region, for this drug only. Principle component analysis agreed with this analysis, showing no population clustering and no patterns emerging. This was expected at such an early time point, only 24 hours after introduction to the diet; any expected effects on development would become obvious after further exposure to the drugs of interest. Based on the results shown, at 24 hours after exposure to one of several Novel Psychoactive Substances or common adulterants, no significant differences were observed. This means that larval age at this point would not be incorrectly estimated due to drug presence and PMI estimations would not be affected.

For ease, on all following sampling days, significant differences ($p < 0.001$) are shown with a star on box plots. ★

Day 2- Results

Larval Length

Initial observations of larval length showed three of the sample populations to have produced larvae, which seemed larger than the control (5EAPB, Pink Panther and Benzofury Beige). A number of larvae seemed in general larger than the control group. Larval lengths ranged from 5.43mm (Paracetamol) to 9.75mm (5EAPB). When tested using a mixed effects model (Figure 85), comparing the lengths across the different diet, significant differences were shown ($F=22.28$, $P<0.0001$). Further analysis to define where the differences lay showed a larger number of differences than seen previously at 24 hours. Data analysis will focus mainly on the differences found between the control and other diet populations as this is of most forensic importance. No larvae feeding on any of the researched diets were shown to be significantly shorter than the control population (mean= 6.811mm, SD=0.3325mm) at 48 hours. Larvae feeding on a number of diets were, however, shown to be significantly longer than those within the control population, 5EAPB, Benzofury Beige, Benzofury Blue, Caffeine, Pink Panther ($P<0.01$) and Blow ($p=0.0117$). Significance values are shown, the reader is directed to Table 10 for data values. All other treatments were shown not to cause significant changes within the larval length. As expected, a large number of populations were shown to be significantly different from one and other. Notably 5EAB, Benzofury Beige and Benzofury Green have a larger range of data points.

Larval Weight

Preliminary observations when sampling larval weight showed an obvious development increase in comparison with 24 hours prior, especially when comparing to the control

population. Four populations immediately showed to be larvae of a larger weight, 5EAPB, Benzofury Beige, Benzofury Blue and Pink Panther. Across all populations, the larval weight ranged from 0.0024g (AMT) to 0.0141g (Pink Panther, Benzofury Blue and Benzofury Beige). A mixed effects model was initially used to determine if results were significantly different. ($F=43.666$, $P<0.0001$). This was investigated further using Tukey pairwise tests. Four drug diets were found to be significantly different to the control population. 5EAPB was the only diet found to increase the larval weight significantly ($P=<0.01$), which had also previously been found to significantly increase larval length. Three diets were found to have significantly reduced weights; AMT ($P=<0.01$), Paracetamol ($P=<0.01$) and Blow ($P=<0.01$). Blow had previously been found to have significantly increased in length at this time point. All other treatments were shown not to cause a difference deemed significant. The range of weight measurements seems larger for most excluding the control population. Data set was normally distributed and uniform in variance.

Principle component analysis was carried out using PC 1 and PC 2, which accounted for 100% of the explained variance (Figure 91).

As explained earlier in this chapter, Hoteling's T^2 statistic at a 5% significance level is shown, the outliers defined by this agree with those selected during the pairwise testing. Development data is beginning to show clustering of individual variables, although with overlap. The control samples can be seen in lower centre of the plot and arguably, the data does appear to already be spread across the plot, suggesting that after 48 hours exposure, the drug additions are having an effect on development.

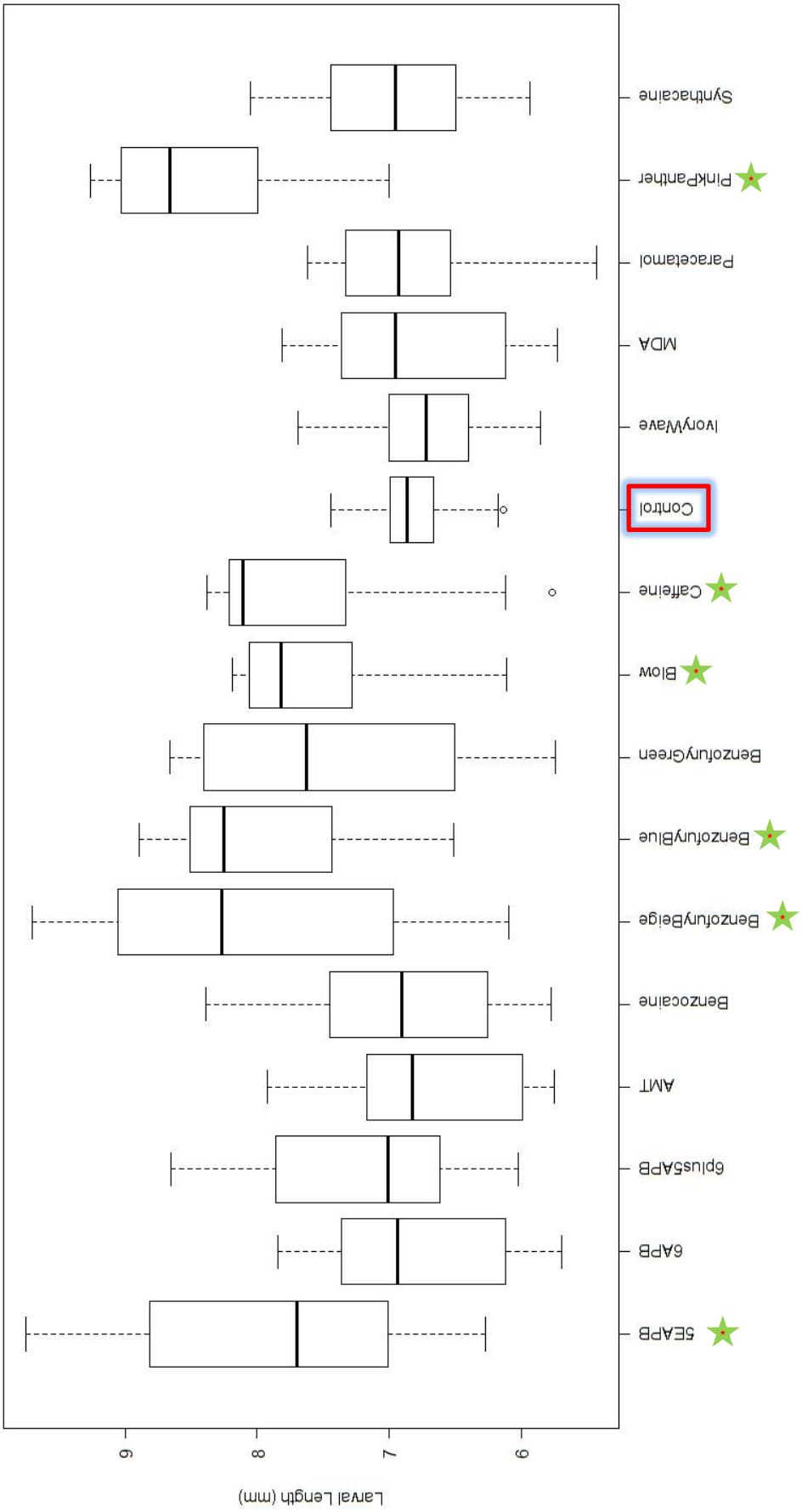


Figure 89: Box plot showing larval length for day 2 across all samples (n=40).

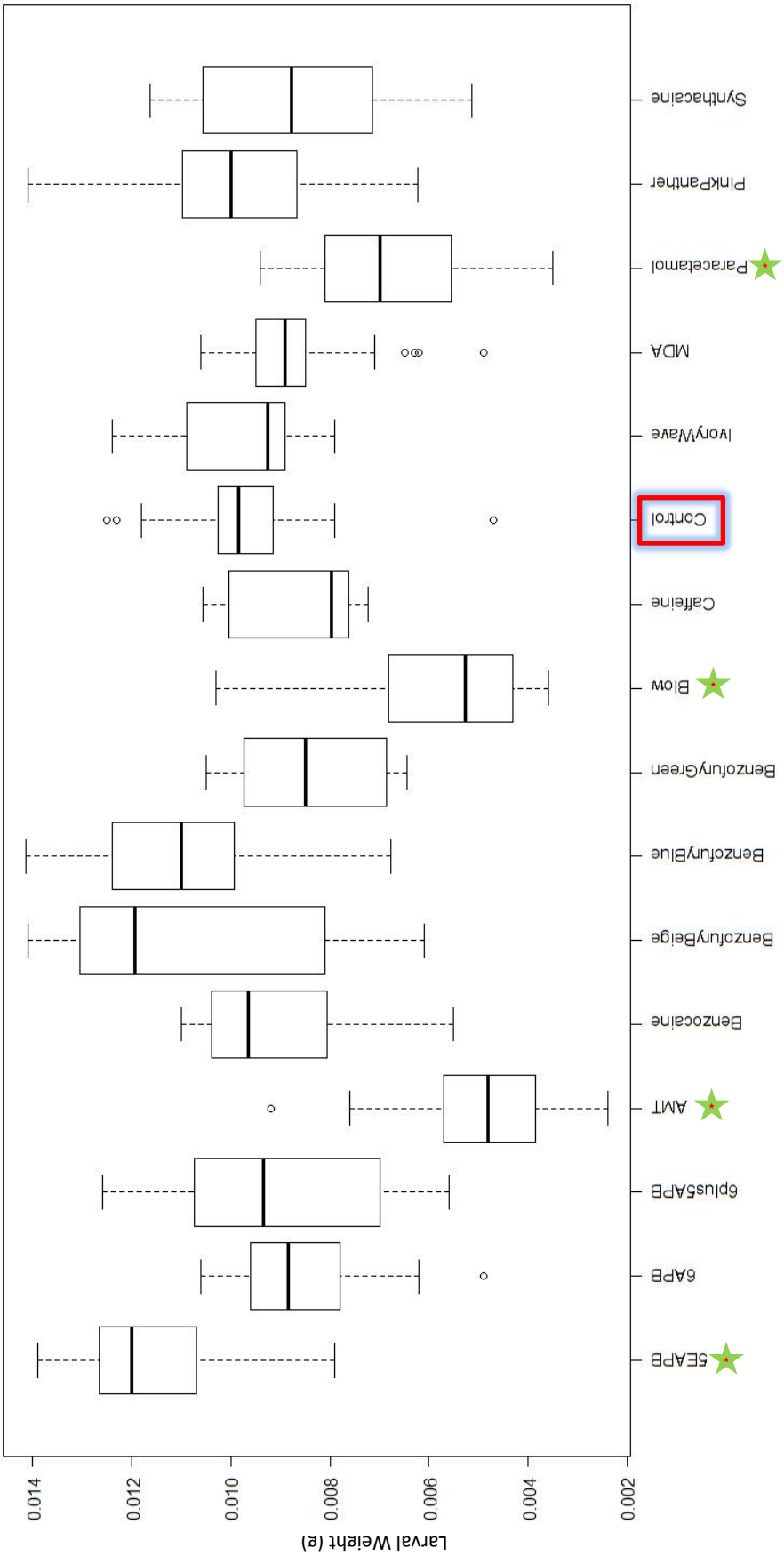


Figure 90: Box plot showing larval weight for day 2 across all samples (n=40).

Table 10: The minimum, maximum and mean lengths and weights (including standard deviation) at 48 hours (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	6.27	9.75	7.8565	1.05	13.30
	Weight (mg)	7.9	13.9	11.6	1.60	13.79
6APB	Length (mm)	5.69	7.84	6.8125	0.65	9.59
	Weight (mg)	4.9	10.6	8.7	1.30	14.94
6+5APB	Length (mm)	6.02	8.65	7.223	0.72	10.02
	Weight (mg)	5.6	12.6	9	2.10	23.33
AMT	Length (mm)	5.75	7.92	6.6878	0.65	9.71
	Weight (mg)	2.4	9.2	5	1.50	30.00
Benzocaine	Length (mm)	5.77	8.39	6.8908	0.72	10.44
	Weight (mg)	5.5	11	9.1	1.50	16.48
Benzofury-Blue	Length (mm)	6.51	8.89	7.9765	0.71	8.87
	Weight (mg)	6.8	14.1	11	1.80	16.36
Benzofury-Green	Length (mm)	5.74	8.66	7.4388	0.98	13.19
	Weight (mg)	6.4	10.5	8.3	1.40	16.87
Benzofury-Beige	Length (mm)	6.09	9.7	8.0403	1.21	15.10
	Weight (mg)	6.1	14.1	10.8	2.60	24.07
Blow	Length (mm)	6.11	8.19	7.598	0.58	7.58
	Weight (mg)	3.6	10.3	5.8	1.90	32.76
Caffeine	Length (mm)	5.76	8.38	7.7433	0.70	9.10
	Weight (mg)	7.2	10.6	8.6	1.20	13.95
Control	Length (mm)	6.13	7.44	6.811	0.33	4.88
	Weight (mg)	4.7	12.5	9.7	1.70	17.53
Ivory Wave	Length (mm)	5.85	7.69	6.7455	0.49	7.23
	Weight (mg)	7.9	12.4	9.7	1.30	13.40
Paracetamol	Length (mm)	5.43	7.62	6.841	0.58	8.44
	Weight (mg)	3.5	9.4	6.8	1.70	25.00
Pink Panther	Length (mm)	7	9.26	8.4338	0.72	8.52
	Weight (mg)	6.2	14.1	9.8	1.80	18.37
Synthacaine	Length (mm)	5.93	8.05	6.9493	0.61	8.83
	Weight (mg)	5.1	11.6	8.7	1.90	21.84
MDA	Length (mm)	5.72	7.81	6.8125	0.65	9.52
	Weight (mg)	4.9	10.6	8.8	1.30	14.77

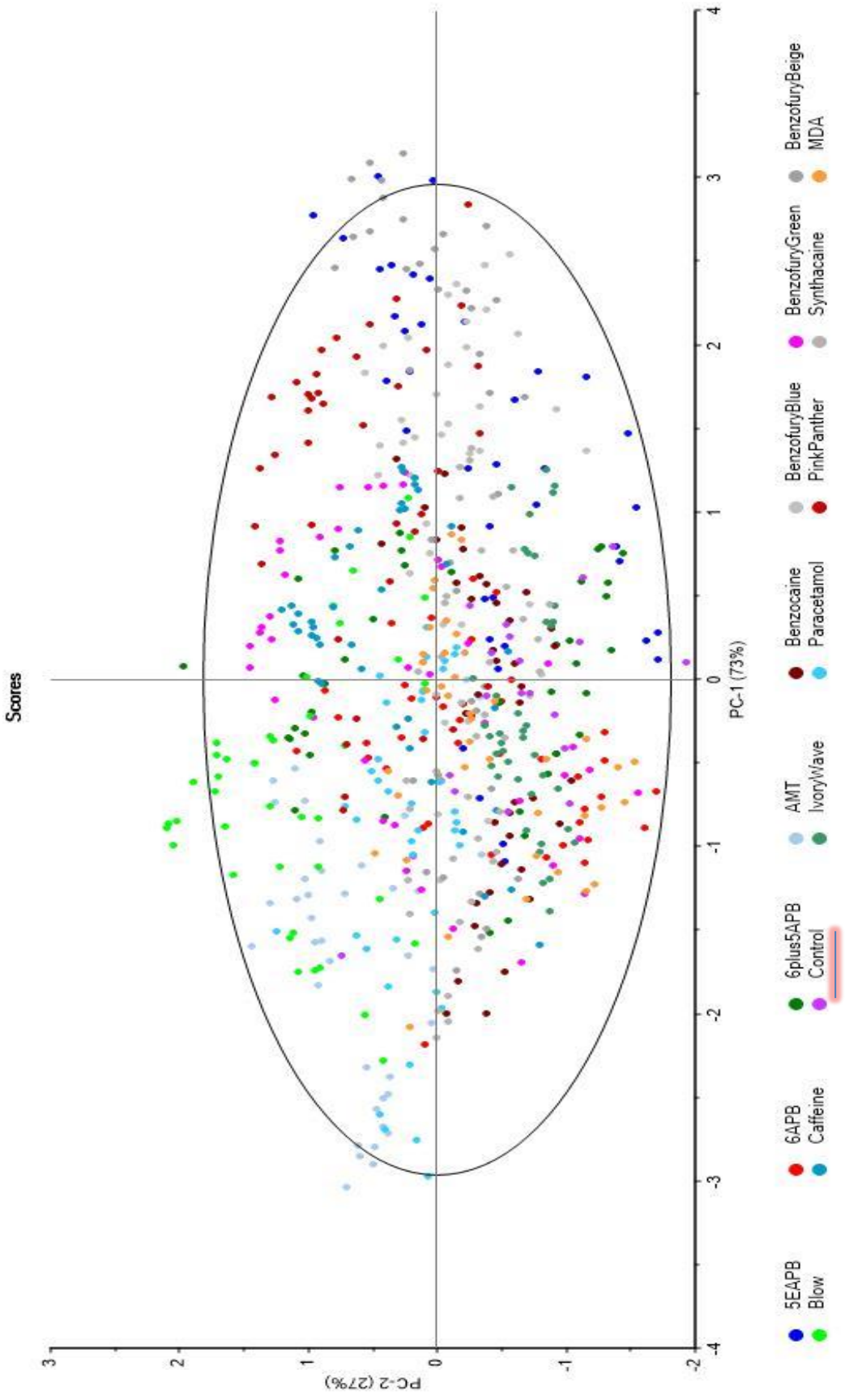


Figure 91: PCA plot showing all drug additions at 48 hours.

Day 2 – Discussion

At 48 hours, insects fed on three of the drugged diets were shown to weigh significantly less than the control population, however these drugs had not caused the same effect on larval length and in one case (Blow) had actually significantly increased length when comparing to the control. All sample populations appear to be developing sufficiently to conclude that they are ingesting the diet provided and therefore the drug also. The population feeding on 5EAPB appear at this time point to have the quickest growth rate and this suggests an increase in response to the drug presence. Further analysis of the data using PCA and utilising 100% of the explained variance at this stage, can begin to separate the different populations. This could be of concern in relation to age estimation of larvae as progression away from expected developmental values is seen. A larger range in data values can also be observed from the boxplots, this could be due to the drug having an effect on some individuals and taking longer in others or perhaps some individuals are yet to feed on the drug or fed later than others.

Day 3

Larval Length

Initial observations regarding larval length at this time point appeared to show drugs accelerating growth (Pink Panther) and also drugs impeding this development (AMT and Paracetamol). Larval lengths ranged from 5.41 mm to 14.9 mm and this larger range would not be expected without an outside influence.

When tested using a mixed effects model comparing the length data acquired, significant differences were found ($F=128.48$, $P<0.0001$). Further analysis showed seven statistically different data ranges. Four of these were shown as significantly larger (5EAPB, Benzofury Beige, Benzofury Blue and Pink Panther) ($P<0.01$) and three significantly smaller (AMT, Blow and Paracetamol) ($P<0.01$). Data values are shown in Table 11. Only one outlier is shown and this is within the data for drug 'Blow'. Data for AMT showed a larger than expected range.

Larval Weight

By this stage, differences in weight between populations were clearly shown. 'Benzofury Beige' and 'Benzofury Blue' were among the largest visually, along with 5EAPB and Pink Panther, whereas 'AMT' and 'Blow' looked smaller than the control population. Larval weights ranged from 0.0058g to 0.0389g.

The mixed effects model showed significant differences were present within the dataset ($F=53.14$, $P<0.0001$). All data were shown to be significantly different from the control group except for 'Pink Panther'. Three drugs resulted in weights significantly larger,

5EAPB, Benzofury Blue and Benzofury Beige ($P<0.01$). All other diets produced significantly lower results ($P<0.01$) (Synthacaine $P=0.0317$). No outliers were shown. Benzofury Blue and Beige showed a larger than expected range. Data set was normally distributed and uniform in variance.

Principle component analysis was performed using PC 1 and 2, together describing 100% of the data. Data clustered defining variables, with some degree of overlap. Variables with lower developmental data appeared to cluster on the left hand side with larger data appearing on the right. Many groups can be easily distinguished. Interestingly, 6APB and MDA show overlapping.

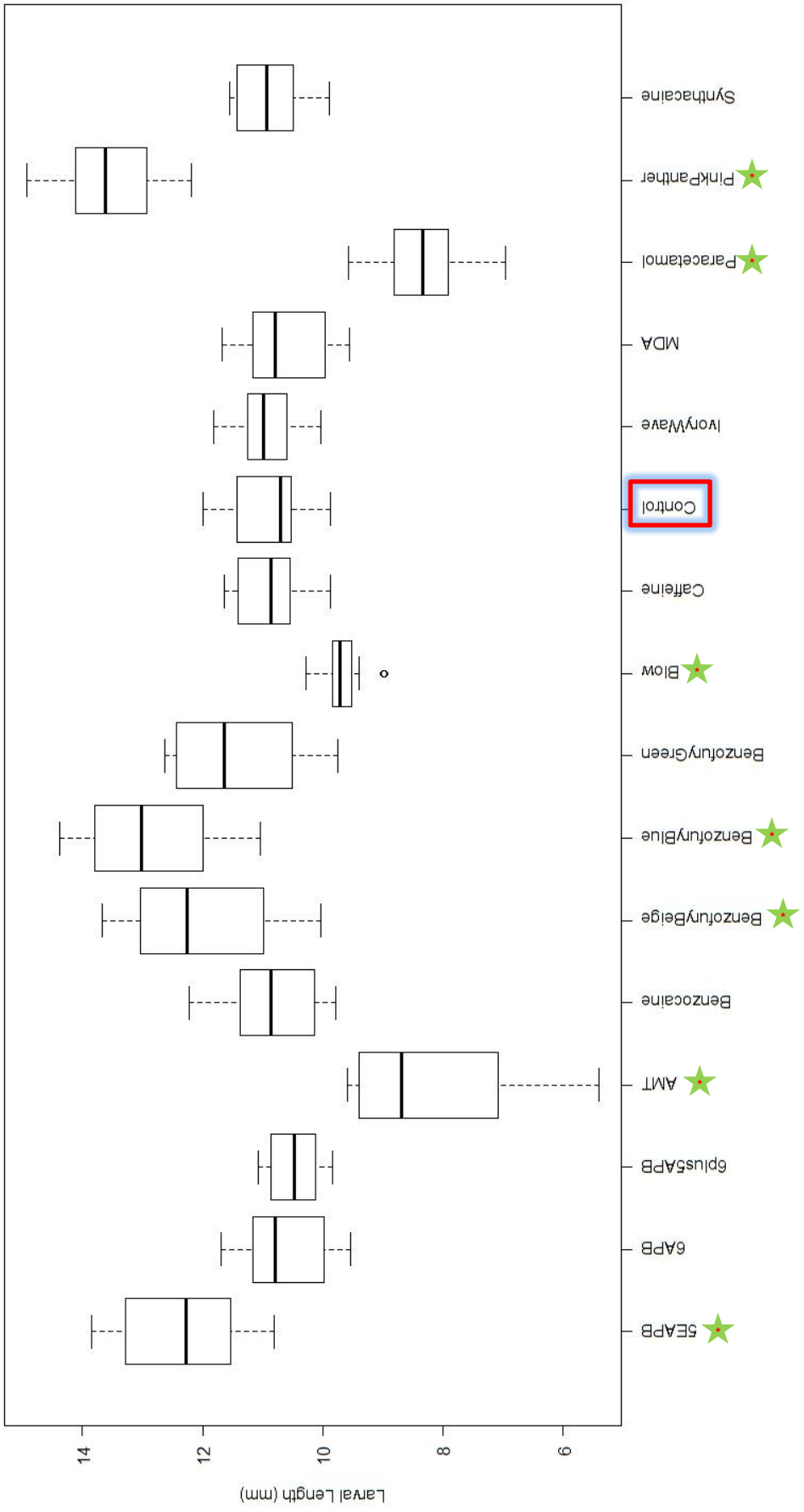


Figure 92: Box plot showing larval length for day 3 across all samples (n=40).

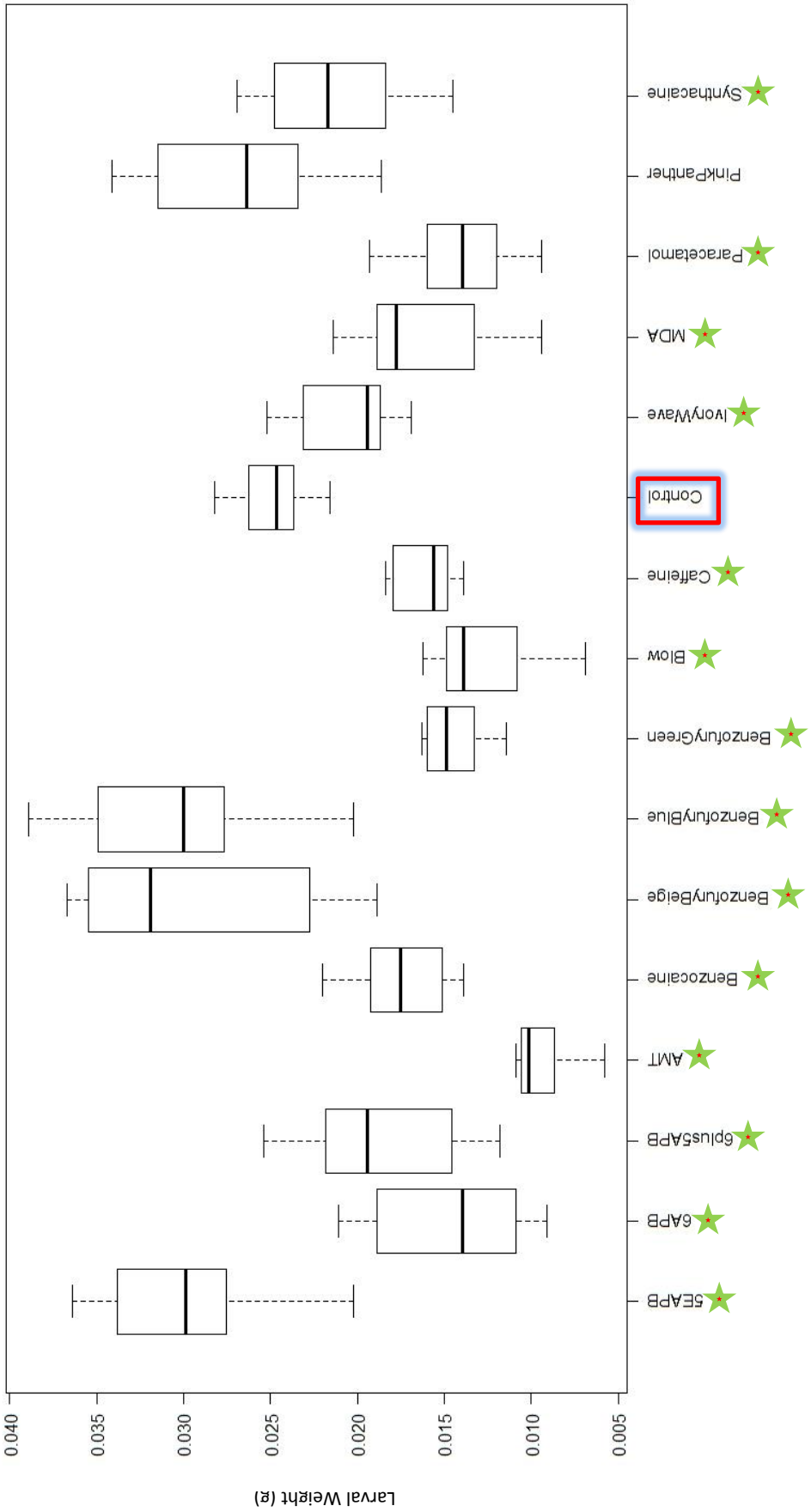


Figure 93: Box plot showing larval weight for day 3 across all samples (n=40).

Table 11: The minimum, maximum and mean lengths and weights (including standard deviation) on day 3 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	10.8	13.84	12.3405	0.96	7.80
	Weight (mg)	20.2	36.4	29.8	4.60	15.44
6APB	Length (mm)	9.54	11.69	10.6468	0.66	6.19
	Weight (mg)	9.1	21.1	14.9	3.90	26.17
6+5APB	Length (mm)	9.84	11.07	10.4798	0.39	3.74
	Weight (mg)	11.8	25.4	18.7	4.10	21.93
AMT	Length (mm)	5.41	9.59	8.3063	1.30	15.63
	Weight (mg)	5.8	10.9	9.7	1.40	14.43
Benzocaine	Length (mm)	9.79	12.23	10.8008	0.73	6.72
	Weight (mg)	13.9	22	17.5	2.40	13.71
Benzofury-Blue	Length (mm)	11.04	14.38	12.9358	1.04	8.07
	Weight (mg)	20.2	38.9	30.4	4.70	15.46
Benzofury-Green	Length (mm)	9.75	12.63	11.4408	0.98	8.54
	Weight (mg)	11.4	16.3	14.6	1.30	8.90
Benzofury-Beige	Length (mm)	10.03	13.67	12.0128	1.21	10.09
	Weight (mg)	18.9	36.7	29.4	6.20	21.09
Blow	Length (mm)	8.97	10.28	9.6875	0.28	2.89
	Weight (mg)	6.9	16.2	12.7	2.80	22.05
Caffeine	Length (mm)	9.87	11.63	10.9085	0.47	4.35
	Weight (mg)	13.9	18.4	16.1	1.50	9.32
Control	Length (mm)	9.87	12	10.8845	0.64	5.88
	Weight (mg)	21.6	28.2	24.7	1.80	7.29
Ivory Wave	Length (mm)	10.03	11.81	10.9598	0.49	4.50
	Weight (mg)	16.9	25.2	20.4	2.60	12.75
Paracetamol	Length (mm)	6.96	9.57	8.3523	0.68	8.11
	Weight (mg)	9.4	19.3	14	2.70	19.29
Pink Panther	Length (mm)	12.19	14.92	13.5718	0.78	5.77
	Weight (mg)	18.6	34.1	27	4.50	16.67
Synthacaine	Length (mm)	9.89	11.55	10.8738	0.53	4.84
	Weight (mg)	14.5	26.9	21.3	3.80	17.84
MDA	Length (mm)	9.557	11.673	10.6468	0.66	6.16
	Weight (mg)	9.4	21.4	15.9	3.70	23.27

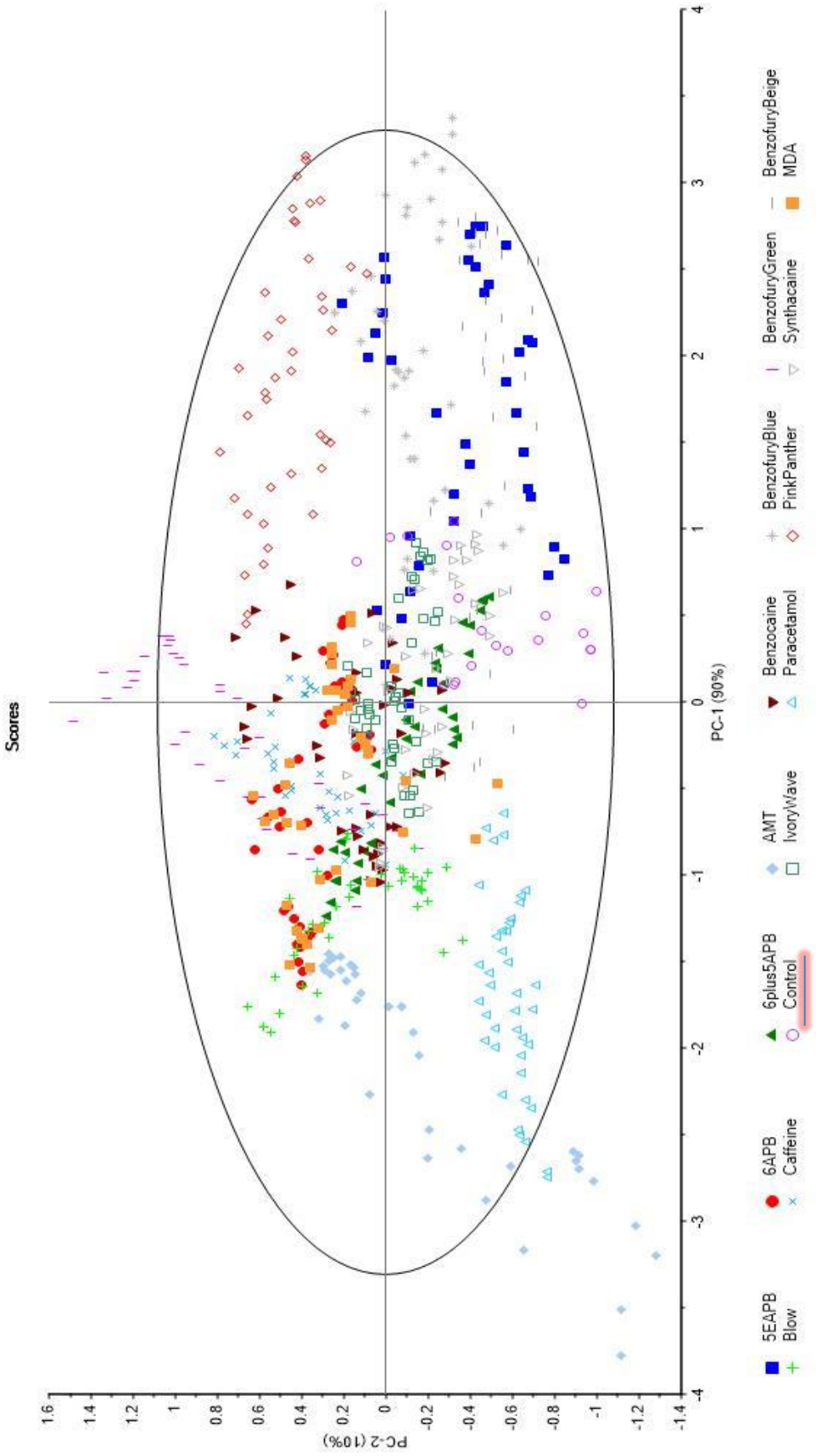


Figure 94: PCA plot showing all drug additions on day 3.

Day 3- Discussion

By this point, it is clear that developmental data were affected by the drug variable as already described.

Benzofury Beige and Benzofury Blue appear to accelerate development; it is suspected that the composition of the three Benzofury batches (Blue, Beige and Green) varies due to the different responses caused.6 APB is thought to be the active ingredient in Benzofury; however, this is currently affecting development quite differently. It could be possible that 6APB is not the active ingredient or another substance will also be found within the drug and is providing the observed response. 6APB is expected to be chemically similar to MDA, currently the response in terms of development, would agree with this. The significant differences shown in the data above could easily allow misidentification of larval age if this situation were to be found at a crime scene.

Some variables appear to show a larger range of length and weight data, it can be hypothesized that this may be caused by the drug amount ingested or perhaps some of the population are actively trying to avoid ingestion of the substances.

Day 4

Larval Length

Observations made during sampling saw the continuation of growth acceleration in Benzofury Beige and Pink Panther, a number of larval samples from the MDA population also seemed larger at this time point. Paracetamol still remained smaller than the control but the growth rate for AMT had increased and samples now looked similar in size to the control population. At day 4 larval lengths ranged between 11.16mm and 20.85mm.

When these results were analysed using a mixed effects model focussed on larval length, significant differences were shown ($F= 88.35$, $P<0.0001$). These differences were investigated further using Tukey tests. All except three drugs were significantly different to the control (6+5APB, AMT and Blow) ($P<0.01$) and of these differences, all except Paracetamol was due to an acceleration of development rate. Paracetamol development is still suppressed. This time point seems to show differences not seen previously with a number of drugs appearing to accelerate growth drastically within the past 24 hours. See Table 12 for all of the data values.

Larval Weight

Populations of Benzofury Beige, Benzofury Blue and MDA stand out in terms of initial observations of a larger larval weight, with paracetamol observed as obviously smaller; this agrees with observations of larval length. Weights of larvae at this stage vary between 0.0349g and 0.117g.

Analysis of day 4 data using a mixed effects model showed significant differences ($F=192.53$, $P<0.0001$), which were investigated further to determine between which variables these differences lay. This showed 5EAPB, 6APB, MDA and Pink Panther to be similar to the control but all other to be significantly different. Only Benzofury Beige and Benzofury Blue were significantly larger ($P<0.01$). MDA had a larger range of values than would be expected. Data set was normally distributed and uniform in variance.

Principle component analysis was performed using PC 1 and 2, which in combination provided 100% explanation of data variance. As seen previously, data clusters dependant on the drug variable. The left-hand side of the plot shows smaller, lighter larvae with the right hand showing longer, larger larvae. The control seems to cluster centrally. As seen in the previous plots MDA and 6APB seem to cluster together nicely. Outliers shown using Hoteling's T^2 statistic at a 5% significance agrees with that shown in the bar plots in Figure 95 and Figure 96.

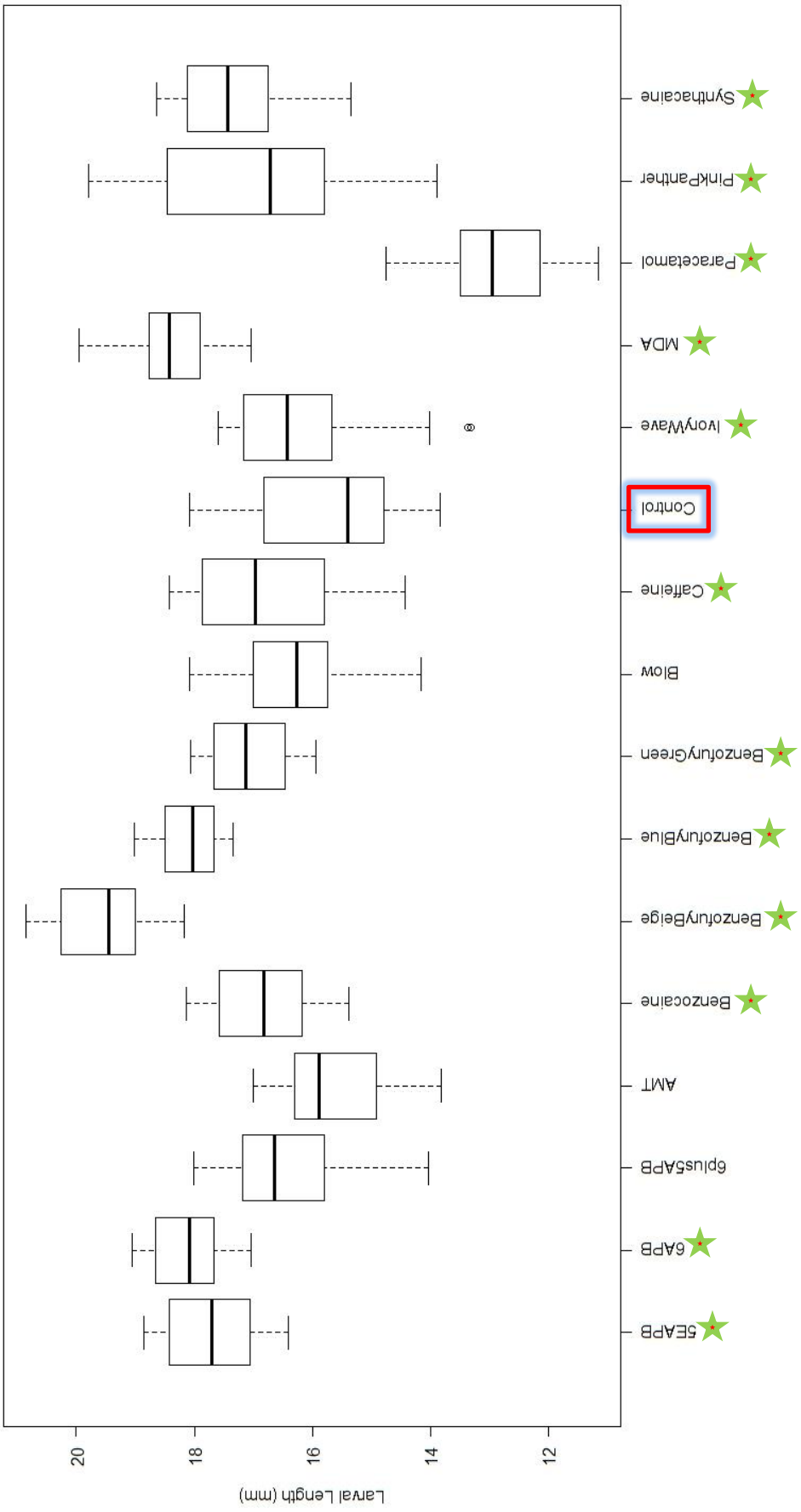


Figure 95: Box plot showing larval length for day 4 across all samples (n=40).

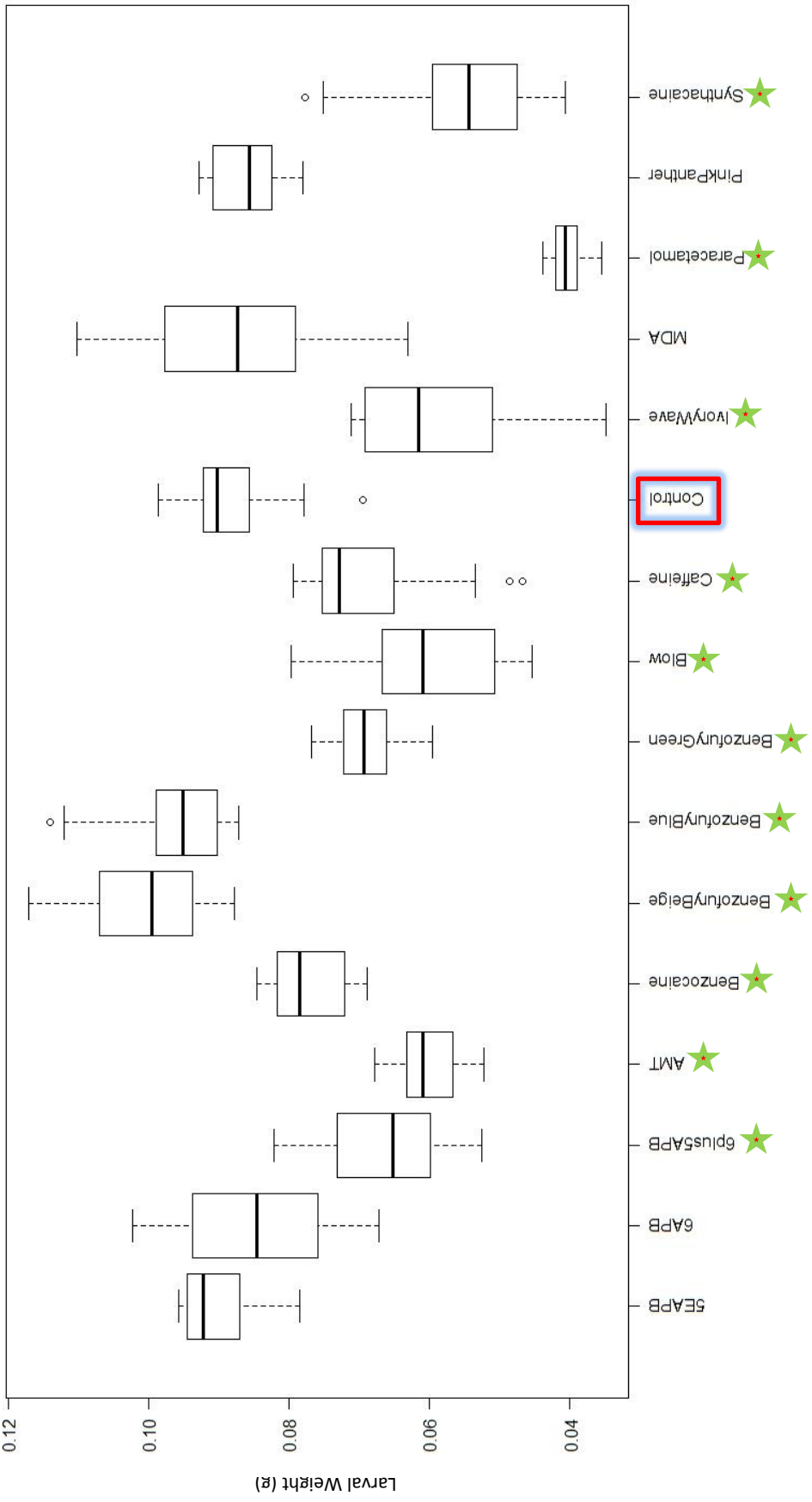


Figure 96: Box plot showing larval weight for day 4 across all samples (n=40).

Table 12: The minimum, maximum and mean lengths and weights (including standard deviation) on day 4 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	16.4	18.85	17.6745	0.80	4.54
	Weight (mg)	78.5	95.7	90.1	5.10	5.66
6APB	Length (mm)	17.03	19.05	18.1548	0.60	3.31
	Weight (mg)	67.1	102.3	84.4	10.40	12.32
6+5APB	Length (mm)	14.04	18.01	16.4115	1.02	6.24
	Weight (mg)	52.6	82.1	66.3	8.20	12.37
AMT	Length (mm)	13.81	17.01	15.612	0.91	5.83
	Weight (mg)	52.2	67.8	60.2	4.10	6.81
Benzocaine	Length (mm)	15.39	18.14	16.846	0.85	5.07
	Weight (mg)	68.9	84.6	77.1	5.20	6.74
Benzofury-Blue	Length (mm)	17.35	19.02	18.074	0.52	2.85
	Weight (mg)	87.2	114	96.4	6.80	7.05
Benzofury-Green	Length (mm)	15.94	18.07	17.0988	0.67	3.89
	Weight (mg)	59.6	76.8	69.1	4.40	6.37
Benzofury-Beige	Length (mm)	18.17	20.85	19.5193	0.79	4.04
	Weight (mg)	87.7	117	100.5	8.60	8.56
Blow	Length (mm)	14.15	18.09	16.3605	1.02	6.23
	Weight (mg)	45.3	79.6	59.7	9.10	15.24
Caffeine	Length (mm)	14.42	18.42	16.7828	1.16	6.89
	Weight (mg)	46.8	79.3	70	7.60	10.86
Control	Length (mm)	13.84	18.09	15.761	1.35	8.55
	Weight (mg)	69.4	98.6	88.1	6.60	7.49
Ivory Wave	Length (mm)	13.32	17.59	16.2583	1.10	6.76
	Weight (mg)	34.9	71.1	59	10.80	18.31
Paracetamol	Length (mm)	11.16	14.75	12.906	0.93	7.18
	Weight (mg)	35.5	43.9	40.4	2.20	5.45
Pink Panther	Length (mm)	13.89	19.8	16.9408	1.78	10.51
	Weight (mg)	78	92.8	86.2	4.60	5.34
Synthacaine	Length (mm)	15.35	18.64	17.2808	0.97	5.62
	Weight (mg)	40.6	77.7	54.3	9.20	16.94
MDA	Length (mm)	17.04	19.96	18.3448	0.73	3.96
	Weight (mg)	63.1	110.2	87.8	11.70	13.33

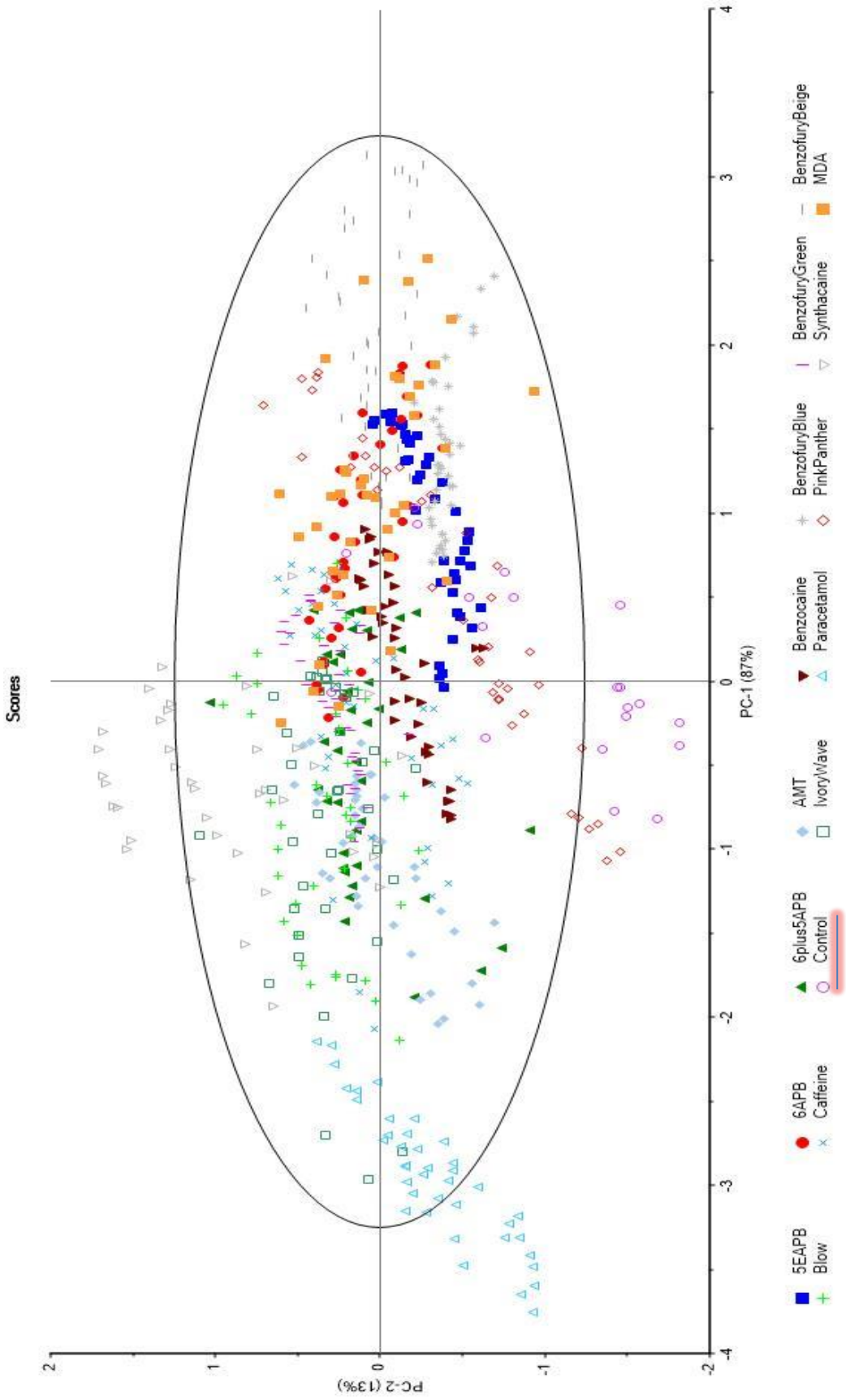


Figure 97: PCA plot showing all drug additions on day 4.

Day 4 - Discussion

Measurements from Day 4 continue to show that developmental of the larvae is influenced by drug presence. Benzofury Beige and Benzofury Blue continue to display an increased growth rate and MDA has now accelerated since the previous sampling day. AMT development was previously supressed and exhibited data ranges similar to that seen in Paracetamol, however, the growth rate has increased over the past 24 hours and bought developmental data in line with the current control population. Before this time point, underestimation of larval age was predicted within the AMT population; larval length and weight is still smaller than seen in the control but the development rate is increasing.

Length and weight data shows a very similar trend when it is compared, this is to be expected as growth accelerates the larval length increases up to a point as does larval weight until the ideal weight is reached, at this point the data should plateau as post feeding initiates.

The significant differences are again shown to be encouraging the necessity of drug factor consideration for accurate PMI estimation.

Day 5

Larval Length

Day 5 showed changes when compared to previous sampling points. All drugs excluding Pink Panther and Paracetamol appear longer than the control samples. Pink Panther appeared very similar in larval length to the control and Paracetamol still appeared to be impeded in development. Larval length ranged from 14.66 mm to 21.61 mm. The control population has a mean larval length of 17.825 mm.

Testing with a mixed effects model showed significant differences across the different drug variables. ($F= 77.2$, $P<0.0001$). Further analysis using Tukey pairwise tests pinpointed these significant differences. They appeared as expected, Pink Panther was not significantly different from the control, all other diets were however, with Paracetamol being the only one that was significantly smaller as opposed to all the other which were statistically larger than the control population ($P<0.01$).

Larval Weight

Weight observations greatly agreed with the previous larval observations. Data values ranged from 0.0427 g to 0.131 g with the control population having a mean of 0.0945 g. A few outliers were shown, all at the lower weight end of the plot.

The mixed effects model showed that the differences found between the variables were significant ($F=53.14$, $P<0.0001$). These differences were found to exist between ten of the tested drugs when compared with the control ($p<0.01$). Of all the drugs shown to be significantly different, only Paracetamol was lower in weight, as seen also in larval length

trends at day five. Five drugs were shown to be similar in weight to the control (6+5 APB, Benzocaine, Pink Panther, Ivory Wave and Synthacaine). Data set was normally distributed and uniform in variance.

Principle component analysis carried out on the data using PC 1 and 2 showed 100% of the explained variance. The plot followed the same trend found across the earlier developmental days where clusters were formed by the different drug variables. Paracetamol, a drug shown in the box plots to have caused impeded growth, is found on the left-hand side, a large number of which are actually shown as outside the 5% significance level shown by Hoteling's T^2 statistic; this provided visualisation of how different these larvae were when compared with the population data as a whole. Data points from control samples are found again towards the centre of the plot, with larger accelerated larval samples found on the right-hand side.

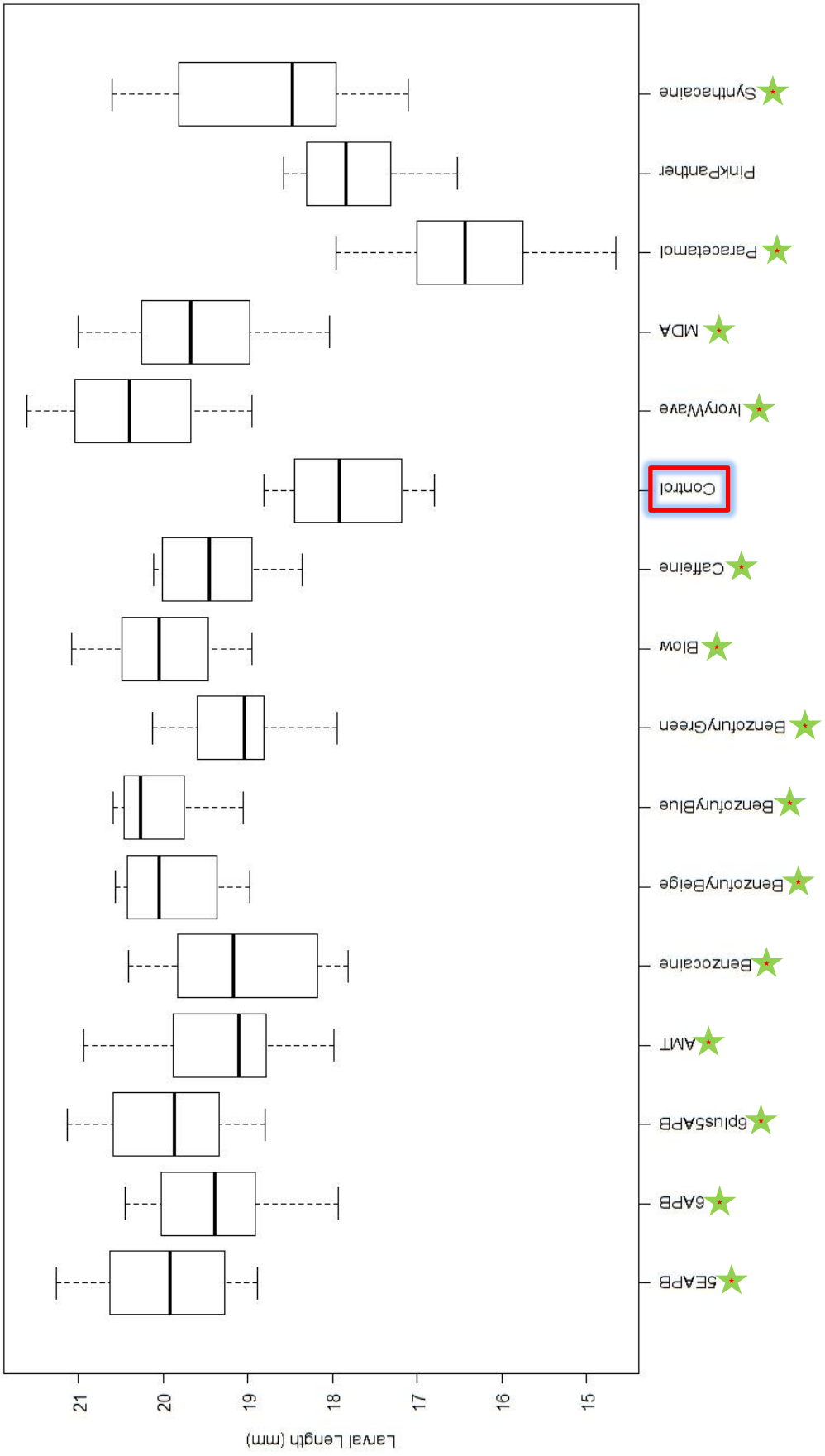


Figure 98: Box plot showing larval length for day 5 across all samples (n=40).

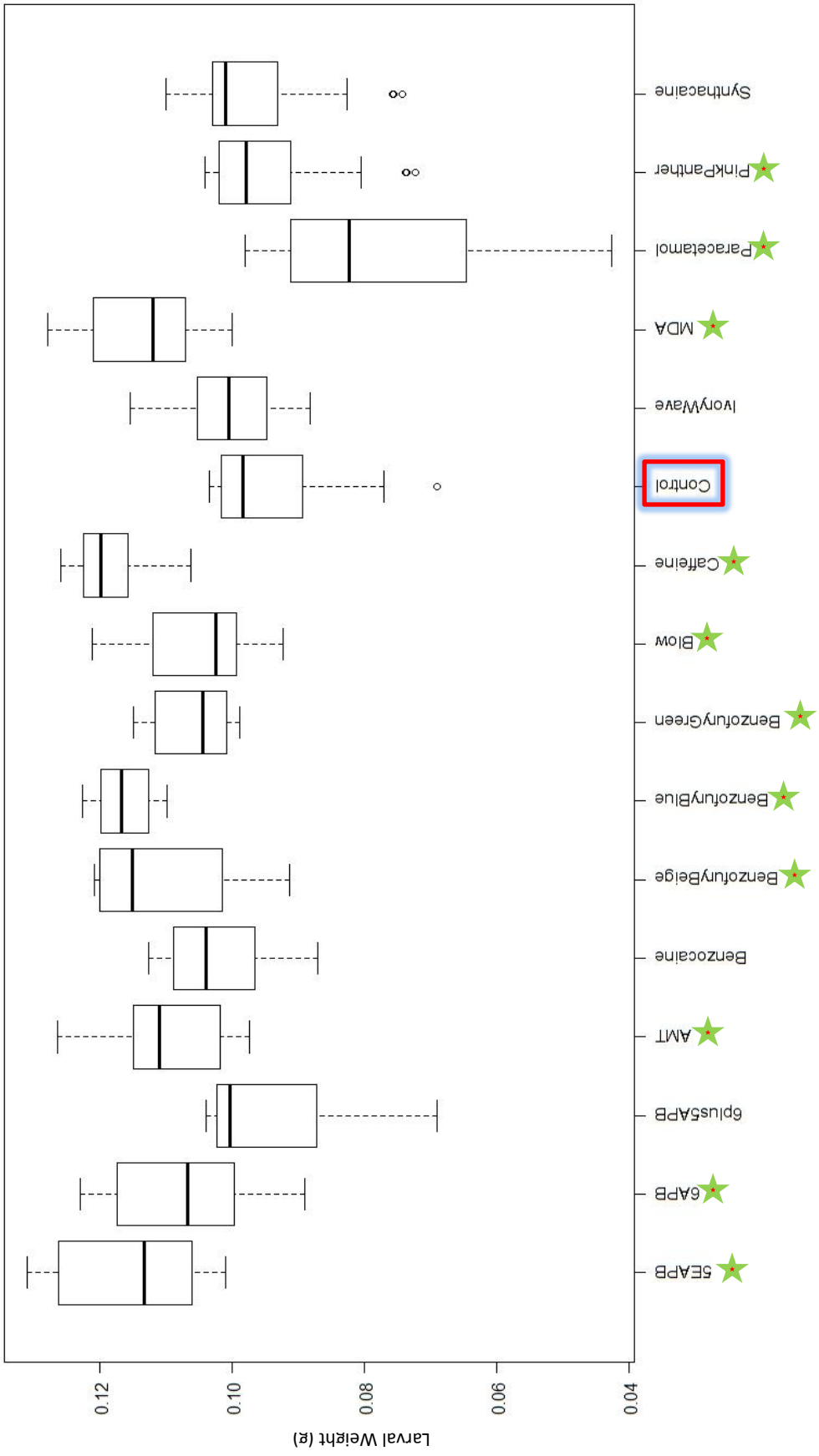


Figure 99: Box plot showing larval weight for day 5 across all samples (n=40).

Table 13: The minimum, maximum and mean lengths and weights (including standard deviation) on day 5 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	18.89	21.26	19.973	0.7325	3.67
	Weight (mg)	101.1	131	115.1	10.2	8.86
6APB	Length (mm)	17.94	20.45	19.3763	0.7644	3.95
	Weight (mg)	89	123	107.9	9.1	8.43
6+5APB	Length (mm)	18.8	21.14	19.9308	0.7439	3.73
	Weight (mg)	69.1	103.9	94.2	11.3	12.00
AMT	Length (mm)	17.99	20.94	19.271	0.8248	4.28
	Weight (mg)	97.4	126.5	110.2	8.3	7.53
Benzocaine	Length (mm)	17.82	20.41	19.102	0.853	4.47
	Weight (mg)	87	112.7	102.4	6.9	6.74
Benzofury-Blue	Length (mm)	19.06	20.59	20.113	0.4086	2.03
	Weight (mg)	109.8	122.7	116.3	4	3.44
Benzofury-Green	Length (mm)	17.95	20.13	19.1333	0.6056	3.17
	Weight (mg)	98.9	115	106	5.6	5.28
Benzofury-Beige	Length (mm)	18.98	20.57	19.9528	0.5189	2.60
	Weight (mg)	91.3	120.8	111	9.6	8.65
Blow	Length (mm)	18.95	21.09	20.033	0.6785	3.39
	Weight (mg)	92.3	121.2	105.8	8.2	7.75
Caffeine	Length (mm)	18.36	20.11	19.4273	0.5404	2.78
	Weight (mg)	106.2	126	118.3	5.4	4.56
Control	Length (mm)	16.8	18.81	17.825	0.6709	3.76
	Weight (mg)	69.1	103.5	94.5	9.8	10.37
Ivory Wave	Length (mm)	18.95	21.61	20.3065	0.8312	4.09
	Weight (mg)	88.2	115.4	100.3	7.2	7.18
Paracetamol	Length (mm)	14.66	17.96	16.4075	0.7304	4.45
	Weight (mg)	42.7	98	79	15.7	19.87
Pink Panther	Length (mm)	16.52	18.58	17.739	0.6445	3.63
	Weight (mg)	72.3	104.1	94.9	9.1	9.59
Synthacaine	Length (mm)	17.11	20.6	18.7895	1.0889	5.80
	Weight (mg)	74.3	110.1	96.8	8.9	9.19
MDA	Length (mm)	18.04	21.01	19.596	0.8606	4.39
	Weight (mg)	100	128	113.7	8	7.04

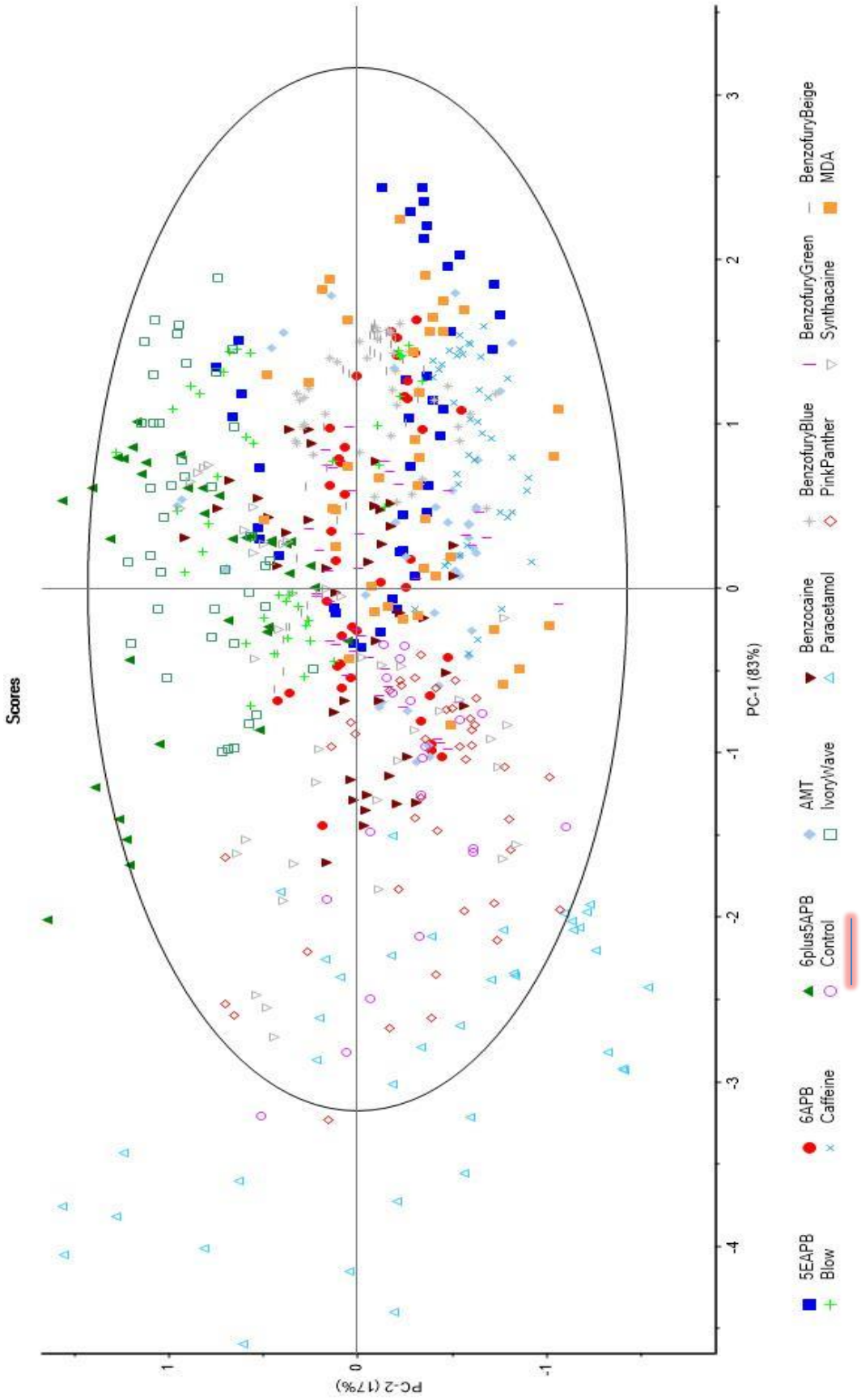


Figure 100: PCA plot showing all drug additions on day 5.

Day 5- Discussion

Trends seen at previous time points are continued at Day 5. Most drugs are shown to have accelerated larval development, and this is attributed to length, weight or in a large amount of cases, both. Development rates previously shown by the larvae ingesting Pink Panther appear to have halted, bringing measurements back in line with the control population, where as before, growth had been shown as accelerated. Paracetamol development continues to be impeded.

Day 6

Larval Length

Initial observations at day six seemed to split samples into two batches. A number of samples were halting the accelerated development seen previously, and were not dissimilar to the measurements of the control population; this included 6+5APB, Benzocaine, Benzofury Green, Ivory Wave, Pink Panther and Synthacaine. Paracetamol was now also showing similar measurements to the control. All other drugs seemed to still be affecting the larvae by accelerating development. Lengths on day 6 ranged between 16.201 mm and 21.38 mm, with control having a mean length of 17.484 mm. No outliers appeared in the samples.

When tested using a mixed effects model comparing larval length, the different diets were shown to be significantly different ($F=90.5$, $P<0.0001$). Tukey tests confirm initial observations of the similarities shown by eight of the drug diets to the control population at day six. All others were significantly larger than the control ($P<0.01$).

Larval Weight

Observations of larval weight during sampling agreed with initial observations shown in larval lengths. Weights at day six ranged from 0.0885 g to 0.1306 g with the control population having a mean weight of 0.0989 g.

As expected, a mixed effects model focussed on larval weight showed significant differences between the larval diets ($F=95.16$, $P<0.0001$). Further analysis showed very similar results to the larval length Tukey test results, with two exceptions. Length for

paracetamol treated samples was shown to be similar to the control in larval length, whereas larval weight was considered significantly larger. 5EAPB had a significantly larger length than the control population but had a weight of similar value. Again no outliers were shown. Data set was normally distributed and uniform in variance.

A scatter graph (Figure 103) was plotted with day six data to show a comparison with the measurements from day one. It shows positive correlation between the two variables described. As before, this is expected due to a general trend of length and weight increasing together. Differences can be seen between Day 1 and day 6 in that individual drug diets can now be seen clustering together instead of as shown in day 1, where the effect of the diet was not yet observed.

Principle component analysis was performed and PC 1 and PC 2 plotted to explain 100% of the sample variance. Data tends to cluster according to the measurement variables, which in turn cause data to cluster for the different drug diets, as seen in day 2 onwards. Blow appears as an outlier within the PCA plot, this is likely due to its larger length and average weight combination. Paracetamol no longer clusters at the edge on the left-hand side and is now positioned closer to the centre. Clusters are still visible, but as some samples are halting development due to the reaching of the ideal larval weight to initiate pupariation, these clusters are not as well defined and increased overlapping is observed. The control is seen further to the left-hand side than in previous plots, this means a larger amount of samples are showing increased developmental measurements in comparison.

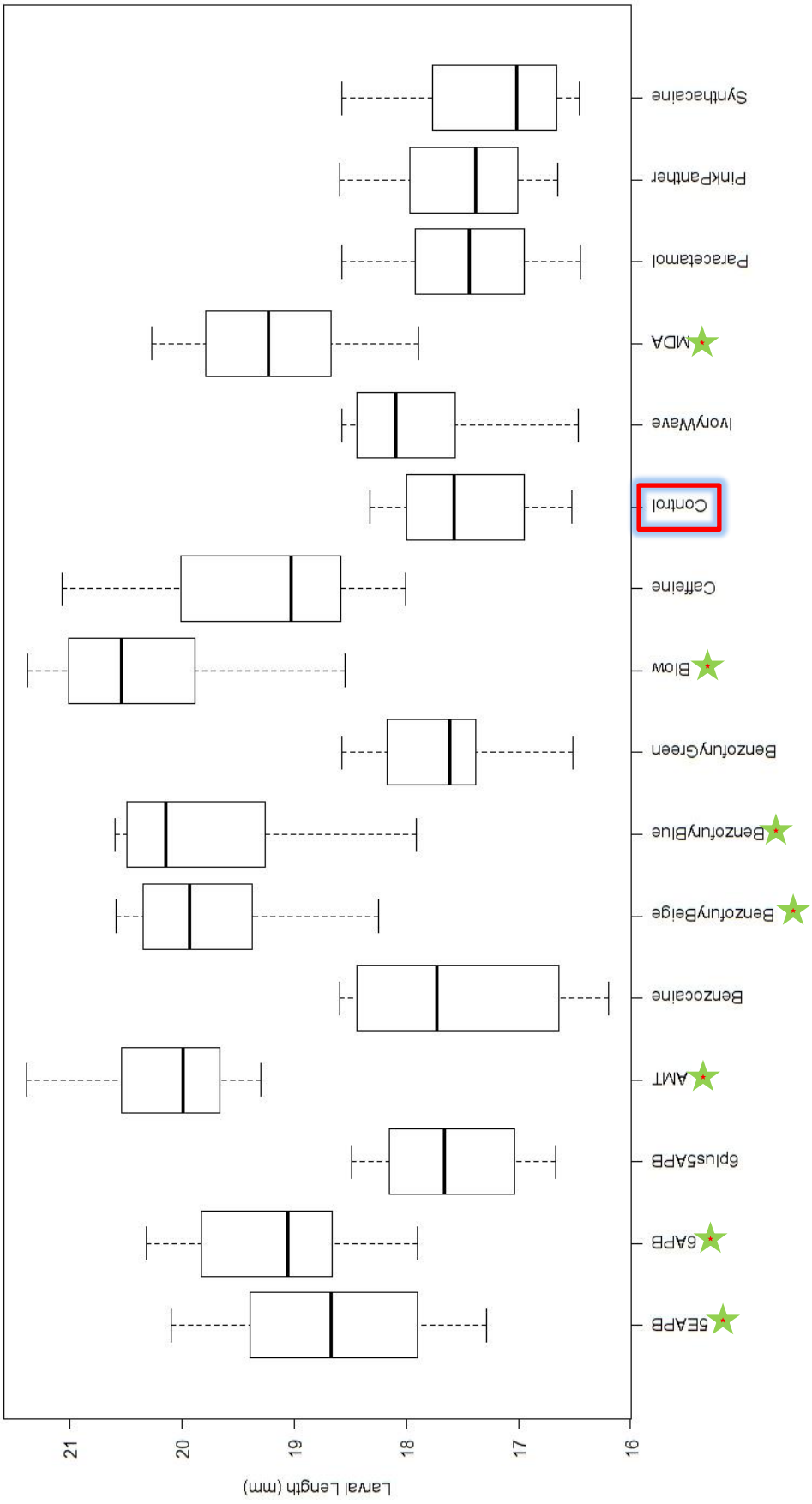


Figure 101: Box plot showing larval length for day 6 across all samples (n=40).

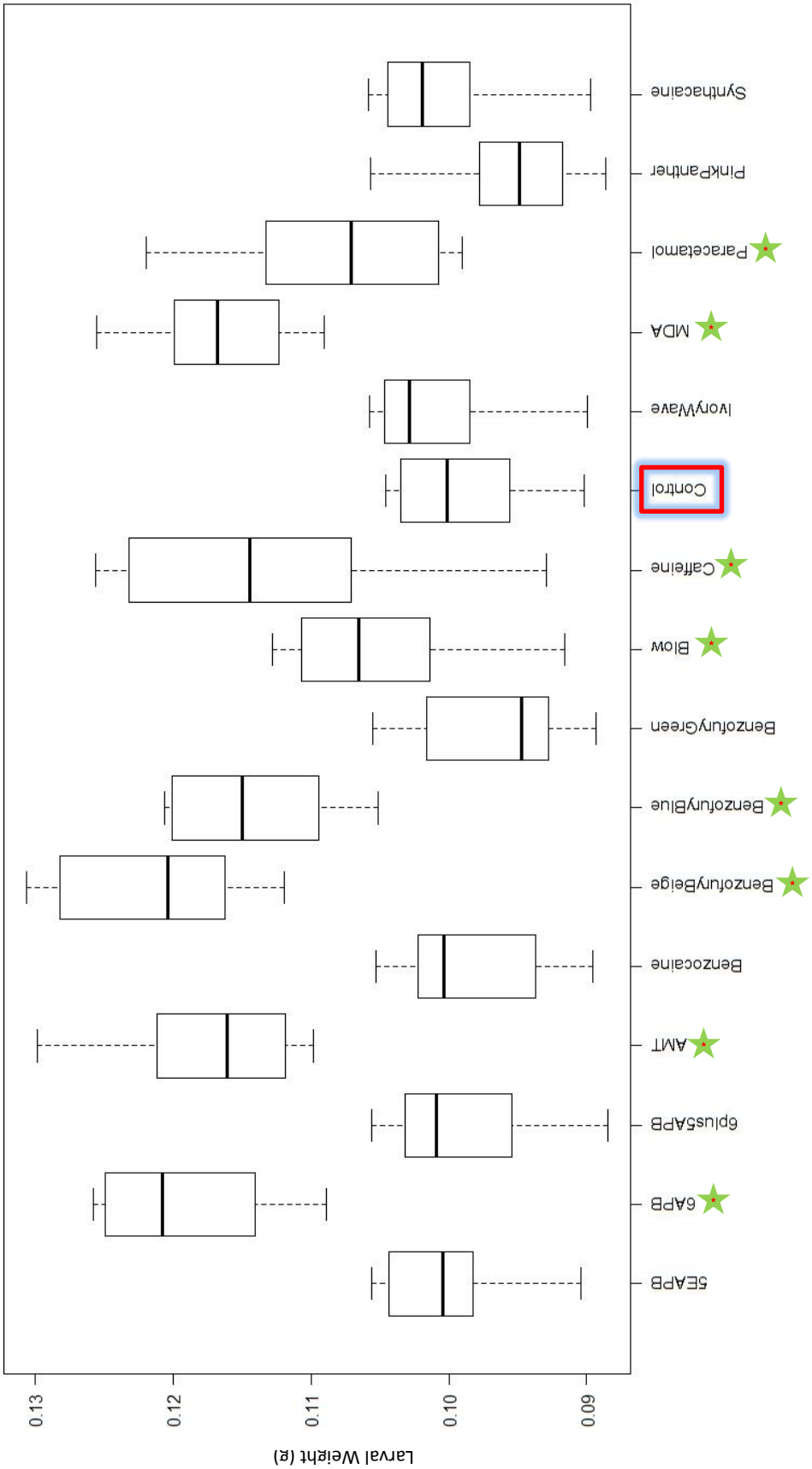


Figure 102: Box plot showing larval weight for day 6 across all samples (n=40).

Table 14: The minimum, maximum and mean lengths and weights (including standard deviation) on day 6 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	17.28	20.09	18.6825	0.872	4.67
	Weight (mg)	90.4	105.6	100.4	3.9	3.88
6APB	Length (mm)	17.9	20.31	19.1455	0.7371	3.85
	Weight (mg)	108.9	125.8	119.3	5.8	4.86
6+5APB	Length (mm)	16.666	18.486	17.6005	0.6158	3.50
	Weight (mg)	88.5	105.6	99.2	5.1	5.14
AMT	Length (mm)	19.29	21.38	20.0843	0.5929	2.95
	Weight (mg)	109.8	129.8	117	6	5.13
Benzocaine	Length (mm)	16.201	18.591	17.635	0.8344	4.73
	Weight (mg)	89.6	105.3	98.3	5.2	5.29
Benzofury-Blue	Length (mm)	17.91	20.59	19.7918	0.7977	4.03
	Weight (mg)	105.1	120.6	114.2	5.6	4.90
Benzofury-Green	Length (mm)	16.512	18.572	17.6828	0.5865	3.32
	Weight (mg)	89.3	105.5	96.8	5	5.17
Benzofury-Beige	Length (mm)	18.25	20.58	19.7983	0.6182	3.12
	Weight (mg)	111.9	130.6	121.7	6.4	5.26
Blow	Length (mm)	18.54	21.37	20.3248	0.08624	0.42
	Weight (mg)	91.6	112.8	105.3	6.2	5.89
Caffeine	Length (mm)	18.01	21.06	19.2848	0.9068	4.70
	Weight (mg)	92.9	125.6	113.9	9.2	8.08
Control	Length (mm)	16.52	18.32	17.484	0.5781	3.31
	Weight (mg)	90.2	104.6	98.9	4.8	4.85
Ivory Wave	Length (mm)	16.466	18.576	17.8943	0.6414	3.58
	Weight (mg)	89.9	105.7	101.2	4.6	4.55
Paracetamol	Length (mm)	16.45	18.57	17.426	0.5928	3.40
	Weight (mg)	99	121.9	108	7.4	6.85
Pink Panther	Length (mm)	16.649	18.589	17.5483	0.5917	3.37
	Weight (mg)	88.6	105.7	95.7	4.9	5.12
Synthacaine	Length (mm)	16.459	18.569	17.2625	0.669	3.88
	Weight (mg)	89.7	105.8	100.6	4.5	4.47
MDA	Length (mm)	17.89	20.27	19.1785	0.7187	3.75
	Weight (mg)	109.1	125.6	116.6	5	4.29

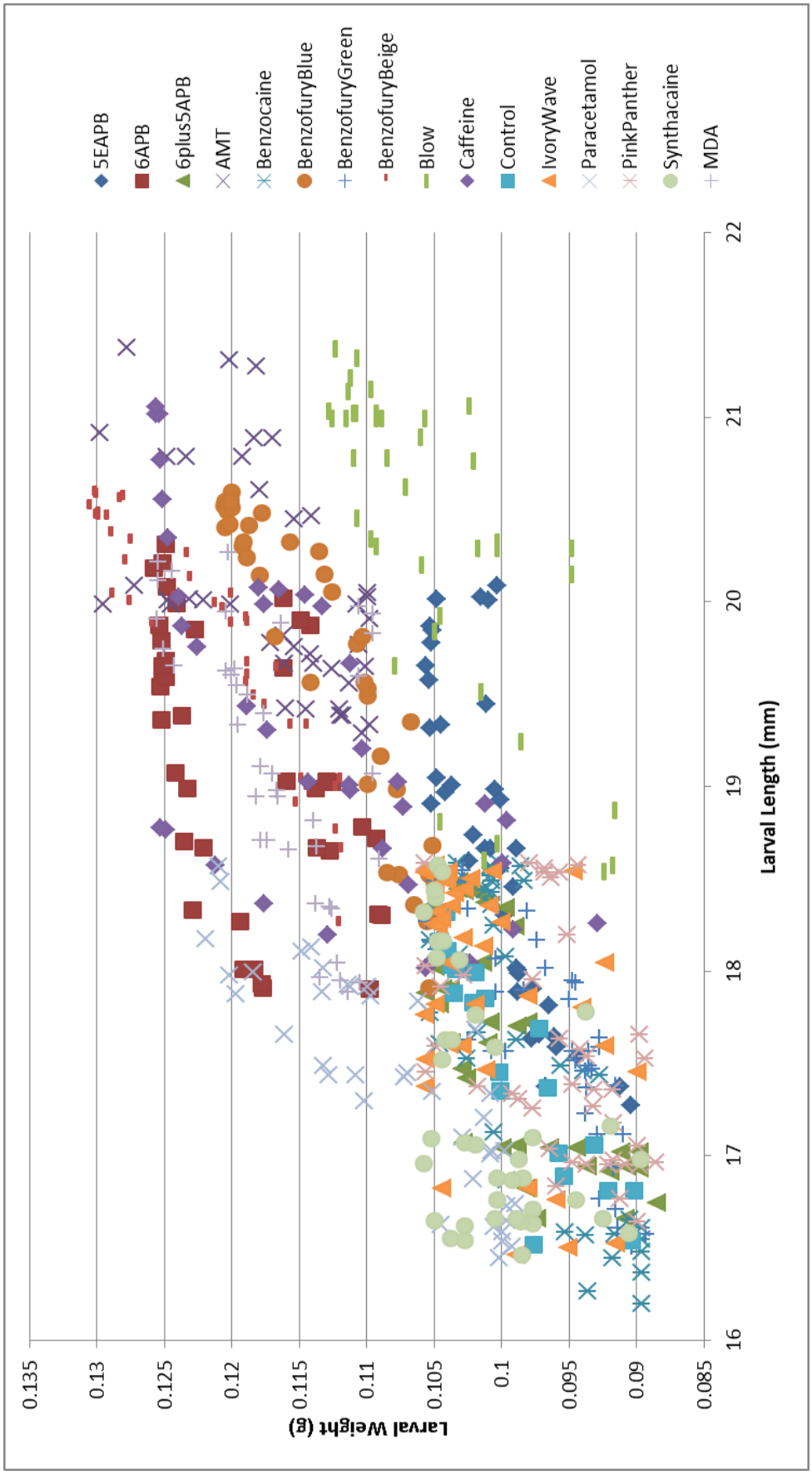


Figure 103: Scatter graph showing linearity of the relationship between larval length and weight.

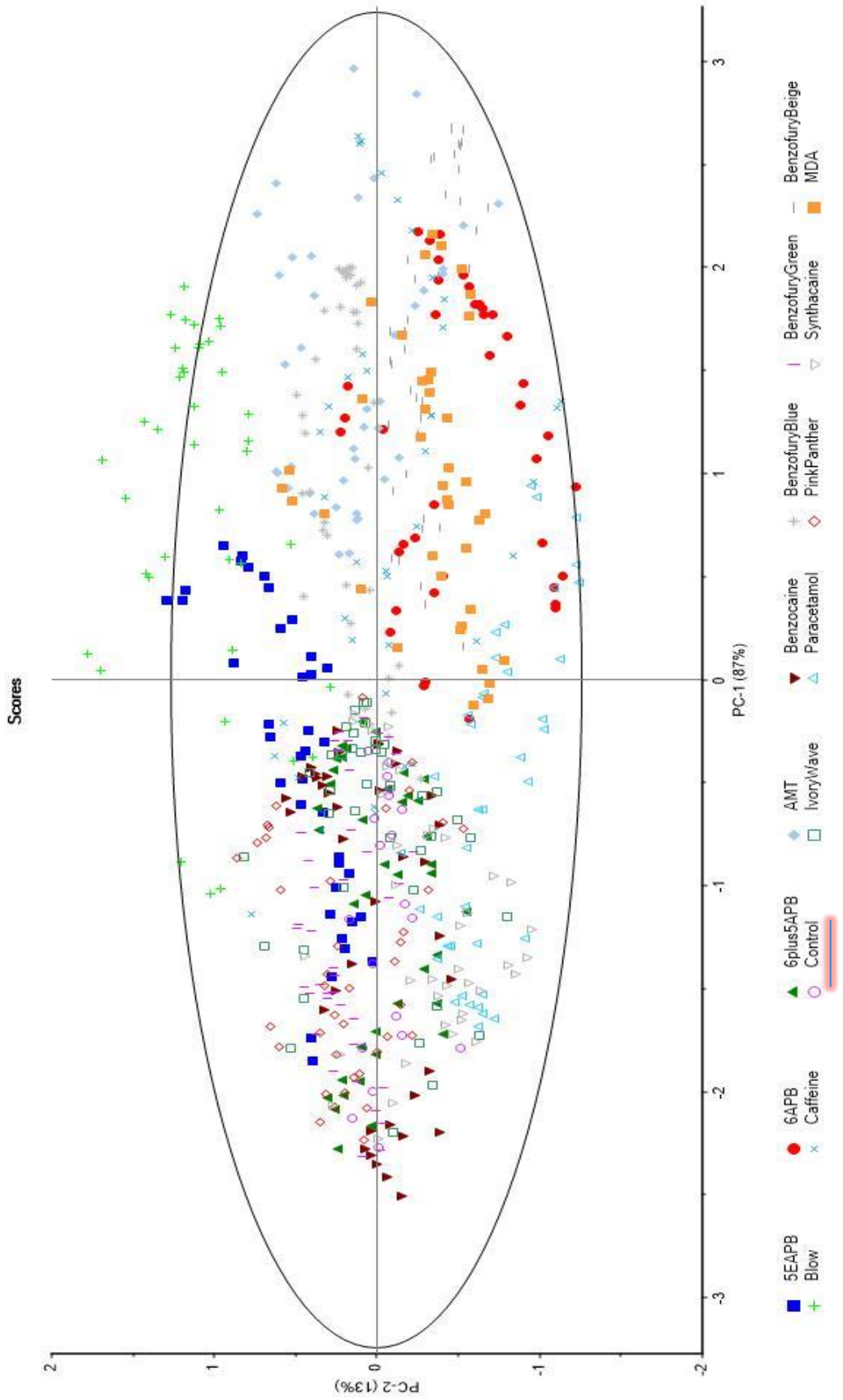


Figure 104: PCA plot showing all drug additions on day 6.

Day 6 - Discussion

The trend on day 6 continues as is expected, with a number of samples previously seen as significantly larger, now reaching the end of the larval stages. Larval length and weight plateaus at this point and appears to be bringing many populations in line with measurements seen in control larvae, but they are in fact ahead of development. Paracetamol measurements have also been brought in line with the control population, but this is because the development rate is increasing. It is possible that the paracetamol impeding the growth in the time points prior to day 6, is now no longer having an effect. AMT is also shown to have increased its developmental rate; at day 4, measurements were shown to be significantly lower than the control, day 6 is now showing AMT to be significantly larger in length and weight than the control. Data is shown to have specific boundaries for each drug variable, which enable quite clear clustering across both PCA, and scatter plots. As post-feeding stage is ending, it is possible there will be further overlapping as weight is lost and larval length shrinks.

Day 7

Larval Length

Initial length observation showed a mix of larval sizes at every population, it was difficult at this point to specify a common theme amongst the measurements of an individual drug, there was however, an increase in wandering. Lengths ranged from 13.98 mm to 20.91 mm, control had a mean value of 16.326 mm. Outliers were shown within the caffeine treatment, however a smaller range was also observed so it is likely data were not evenly spread.

A mixed effects model focussed on larval length continued to show statistically different results for the different drug treatments ($F=105.81$, $P<0.0001$). Tukey tests enabled the breakdown of this result. Benzofury Green, Ivory Wave, Paracetamol, Pink Panther and Synthacaine all showed statistically similar results to the control population. Looking at the previous trend observed with each of these populations (except Paracetamol), it can be suggested that this is due to the shrinkage experienced before arrival at pupariation. Paracetamol is likely to be statistically similar due to the delays experienced during the first few days of the larval stages. All other drug variables were statistically different ($P<0.01$) from the control. Two drugs showed significantly lower measurements, 6+5APB and Benzocaine. This again can be attributed to larval shrinkage and the initiation of pupariation. All others were significantly larger than the control.

Larval Weight

Weight observations showed again, a mix where a clear trend could not be determined. Larval weights ranged from 0.0604 g to 0.1242 g, control had a mean weight of 0.0933g.

A mixed effects model determined diet treatments were significantly different from the control when focussed on larval weight ($F=112.27$, $P<0.0001$). Tukey tests determined that these differences were very similar to the observations of larval length at day 7. Benzofury Green, 6+5APB, Caffeine, Ivory Wave and Synthacaine were observed to be similar in weight to the control and for all of these diets, considering the pattern they have followed, it would be suggested this is due to post-feeding and initiation of pupariation. Caffeine length was shown to be significantly larger than the control population; the outliers visible in the boxplot in Figure 106 may have encouraged a result lower in weight than expected due to skewed data. All other diets are shown to be significantly different to the control ($P<0.01$), all showing an increased weight except Pink Panther and Benzocaine. Benzocaine is also shown to have a length less than the control and it is hypothesized that this is due to early onset pupariation. Pink Panther has a length similar to the control but a reduced weight, suggesting the same conclusion. Data set was normally distributed and uniform in variance.

Principle component analysis plotted with PC 1 and PC2 showing 100% of data variance showed clustering as expected. A trend is followed where smaller, lighter larvae are on the left-hand side, increasing in length and weight the further towards the right. Some samples of Pink Panther appear as outliers, most likely due to their lower than expected weight when considering their length, due to imminent pupariation. Control samples are showing more centrally again.

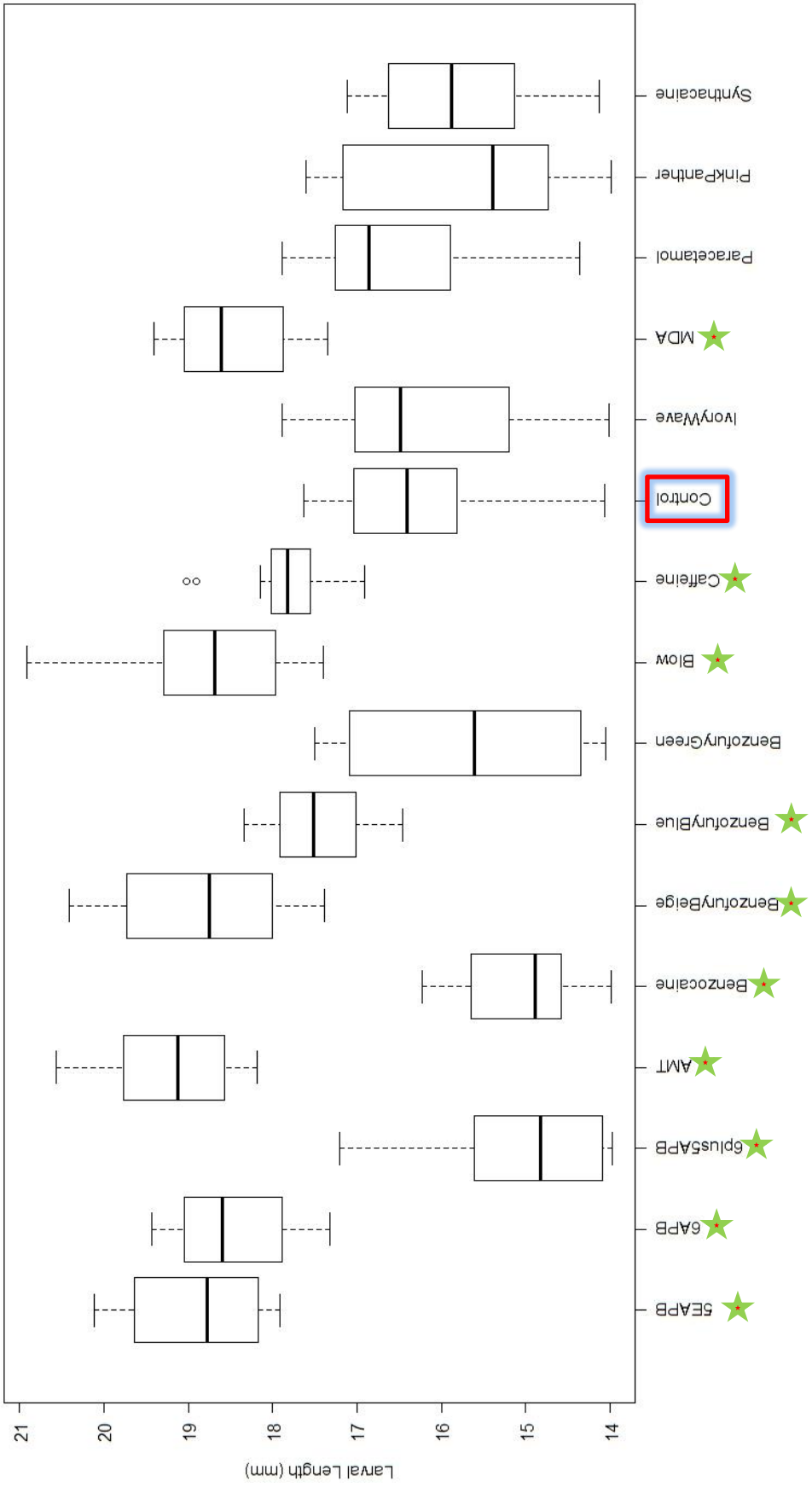


Figure 105: Box plot showing larval length for day 7 across all samples (n=40).

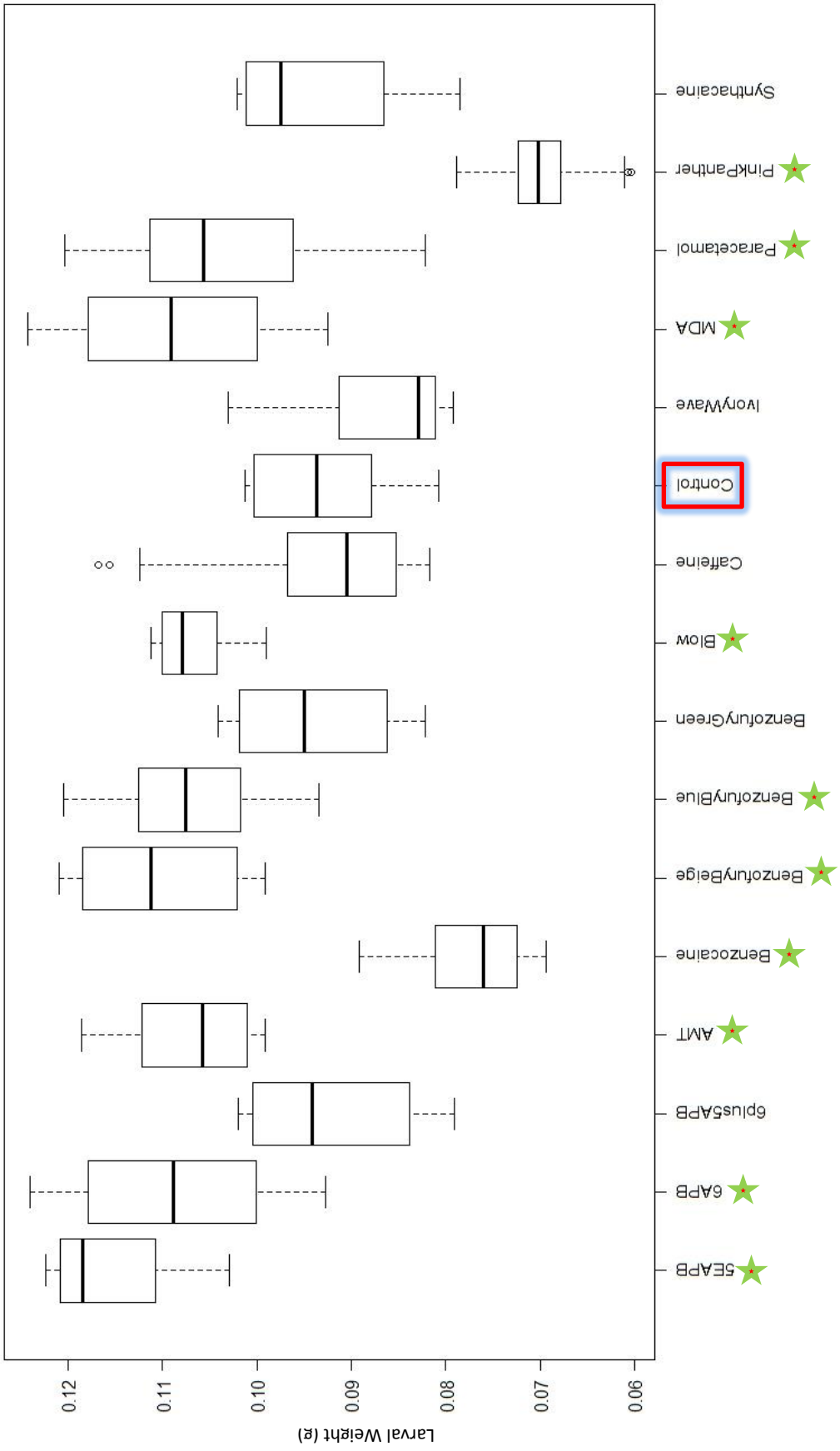


Figure 106: Box plot showing larval weight for day 7 across all samples (n=40).

Table 15: The minimum, maximum and mean lengths and weights (including standard deviation) on day 7 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	17.92	20.12	18.8358	0.7421	3.94
	Weight (mg)	102.9	122.4	115.8	6.5	5.61
6APB	Length (mm)	17.32	19.43	18.4913	0.657	3.55
	Weight (mg)	92.7	124	109.3	9.4	8.60
6+5APB	Length (mm)	13.98	17.21	15.0718	1.0683	7.09
	Weight (mg)	79.1	102	92.2	8.3	9.00
AMT	Length (mm)	18.19	20.57	19.181	0.6963	3.63
	Weight (mg)	99.1	118.6	106.8	5.8	5.43
Benzocaine	Length (mm)	13.99	16.23	15.0595	0.705	4.68
	Weight (mg)	69.4	89.2	77.2	5.9	7.64
Benzofury-Blue	Length (mm)	16.46	18.34	17.477	0.5188	2.97
	Weight (mg)	93.4	120.5	107.3	6.8	6.34
Benzofury-Green	Length (mm)	14.05	17.5	15.8008	1.3347	8.45
	Weight (mg)	82.2	104.1	94.3	7.7	8.17
Benzofury-Beige	Length (mm)	17.38	20.41	18.816	0.9035	4.80
	Weight (mg)	99.1	120.9	110.3	8	7.25
Blow	Length (mm)	17.4	20.91	18.783	1.0104	5.38
	Weight (mg)	99	111.2	106.6	3.6	3.38
Caffeine	Length (mm)	16.91	19.02	17.7953	0.3976	2.23
	Weight (mg)	81.7	116.8	93.3	9.8	10.50
Control	Length (mm)	14.06	17.63	16.326	0.94	5.76
	Weight (mg)	80.7	101.3	93.3	6.9	7.40
Ivory Wave	Length (mm)	14.01	17.89	16.206	1.128	6.96
	Weight (mg)	79.2	103	86.5	7	8.09
Paracetamol	Length (mm)	14.36	17.89	16.5595	0.9639	5.82
	Weight (mg)	82.2	120.4	104.1	10.3	9.89
Pink Panther	Length (mm)	13.99	17.61	15.7335	1.2857	8.17
	Weight (mg)	60.4	78.8	69.9	4.6	6.58
Synthacaine	Length (mm)	14.13	17.11	15.8453	0.865	5.46
	Weight (mg)	78.5	102.1	94	8.1	8.62
MDA	Length (mm)	17.343	19.407	18.4913	0.6538	3.54
	Weight (mg)	92.5	124.2	109.3	9.4	8.60

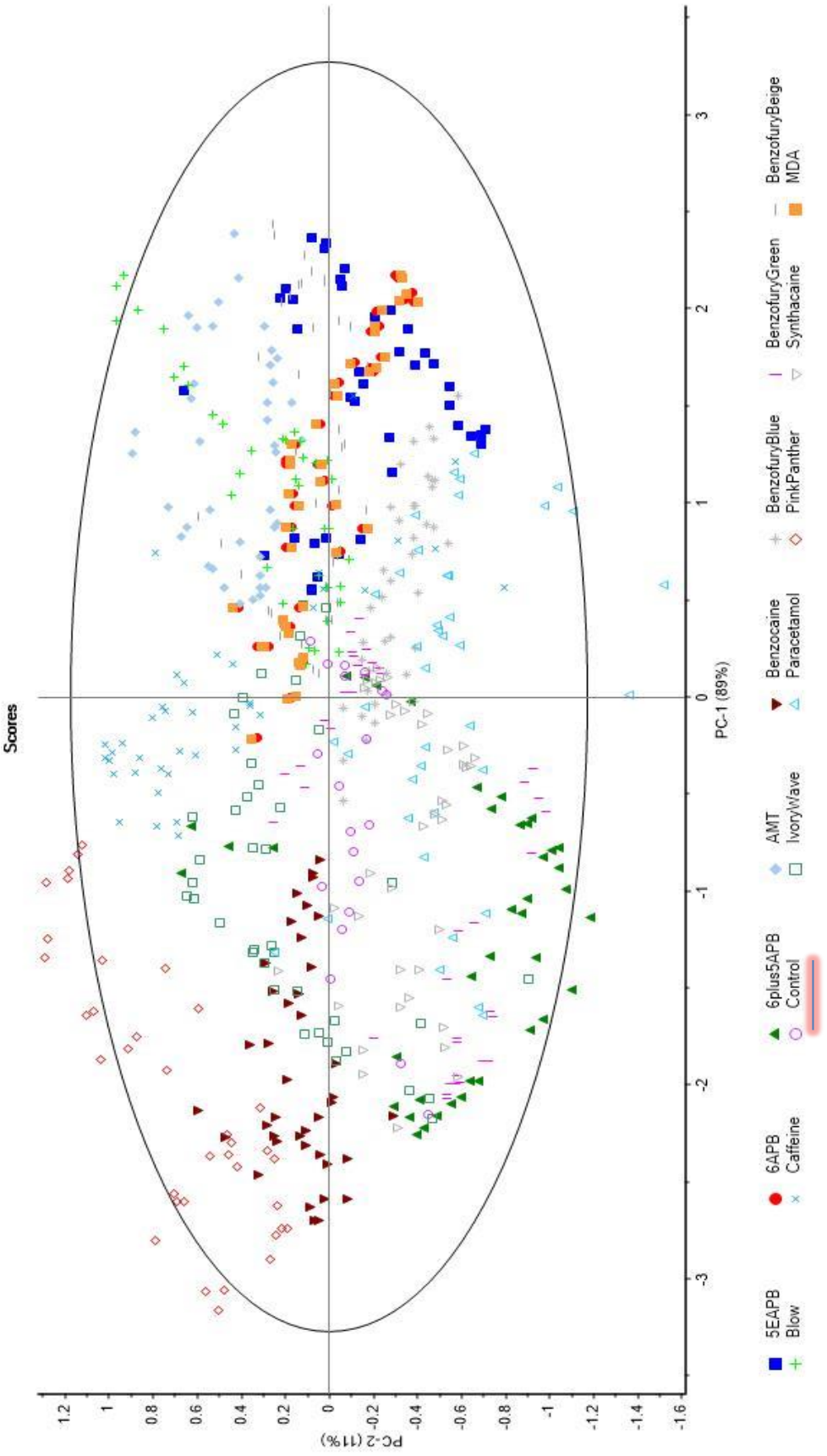


Figure 107: PCA plot showing all drug additions on day 7.

Day 7 –Discussion

Day 7 displays a select few drug variables continuing to show accelerated growth (5EAPB, 6APB, AMT, Benzofury Beige, Benzofury Blue, Blow and MDA), a few samples within the researched compounds had also reached a stage close to pupariation. This was determined by larval behaviour (movement away from the food source) and also the shrinkage observed within measurements (Benzofury Green, Ivory Wave, Pink Panther and Synthacaine). Paracetamol appeared to continue increasing in weight although larval length was not dissimilar to the control measurements.

Day 8

When this sampling point was reached, a number of drug treatments had pupariated. These samples are not included in developmental analysis from this point onwards; 6+5APB, Benzocaine, Benzofury Beige, Benzofury Blue, Benzofury Green, Ivory Wave, Pink Panther and Synthacaine. The presence of these drugs has meant earlier than expected pupariation, this could cause over estimation of post mortem interval, as the larvae would be considered later in their developmental cycle.

Larval Length

Initial observations showed no real difference in larval length between the remaining drug treatments with perhaps the exception of 5EAPB, which appeared slightly smaller. Larval length ranged from 13.1 mm to 18.12 mm with the control population showing a mean of 16.5415 mm.

Mixed effects modelling showed that there was indeed still significant differences between the samples ($F=58.23$, $P<0.0001$). Tukey testing showed only two of the treatments were actually significantly different, 5EAPB ($P<0.01$) and Blow ($P=0.0383$). 5EAPB was smaller, again it is expected that this is a result of shrinkage. Blow was shown to be longer in length than the control diet. All other diets at this time point were similar to the control. A number of outliers are shown in the AMT treated diet.

Larval Weight

Initial observations of larval weight at this time point showed limited differences; perhaps Paracetamol larvae were slightly larger than the other populations. Weights ranged from 0.0698 g to 0.1154 g, the control population had a mean weight of 0.0873 g.

Mixed effects modelling showed significant differences to be found amongst the remaining samples ($F=8.01$, $P<0.0001$). Tukey tests were able to pinpoint these differences to four samples. 6APB was shown to be significantly smaller ($P=0.00575$), Caffeine ($P=0.01894$) and MDA ($P=0.01126$) were also both shown to be lighter. Paracetamol was shown to be significantly larger ($P<0.001$), this was the most significant difference observed. Data set was normally distributed and uniform in variance.

Principle component analysis used PC 1 and PC 2 showing 100% of the sample variance. Samples were on a whole, more mixed but individual sample patterns could still be observed. 5EAPB was identified as being different than expected using Hoteling's T^2 statistic at a 5% significance level; which is due to the few smaller larvae observed within this population.

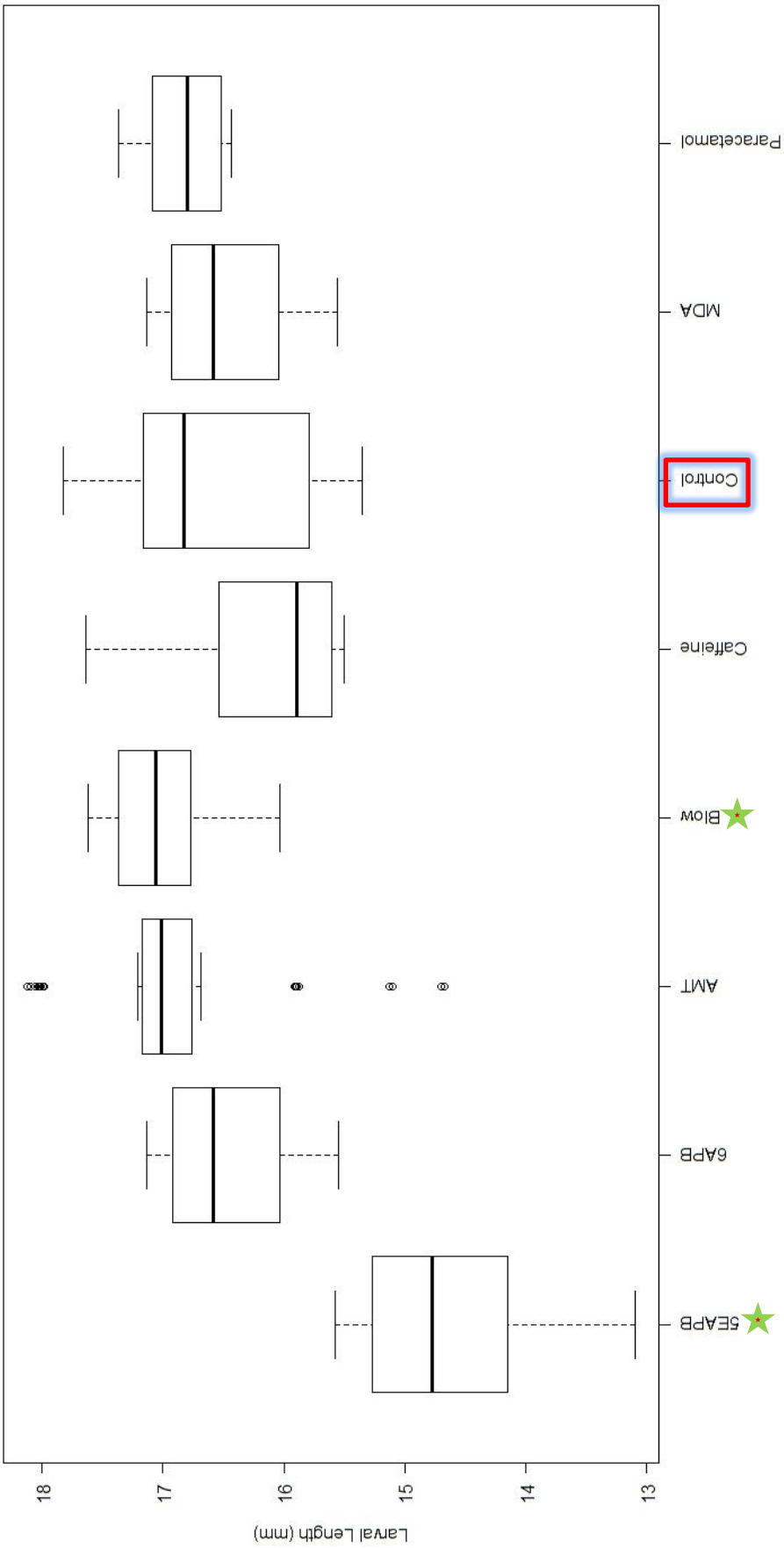


Figure 108: Box plot showing larval length for day 8 across all samples (n=40).

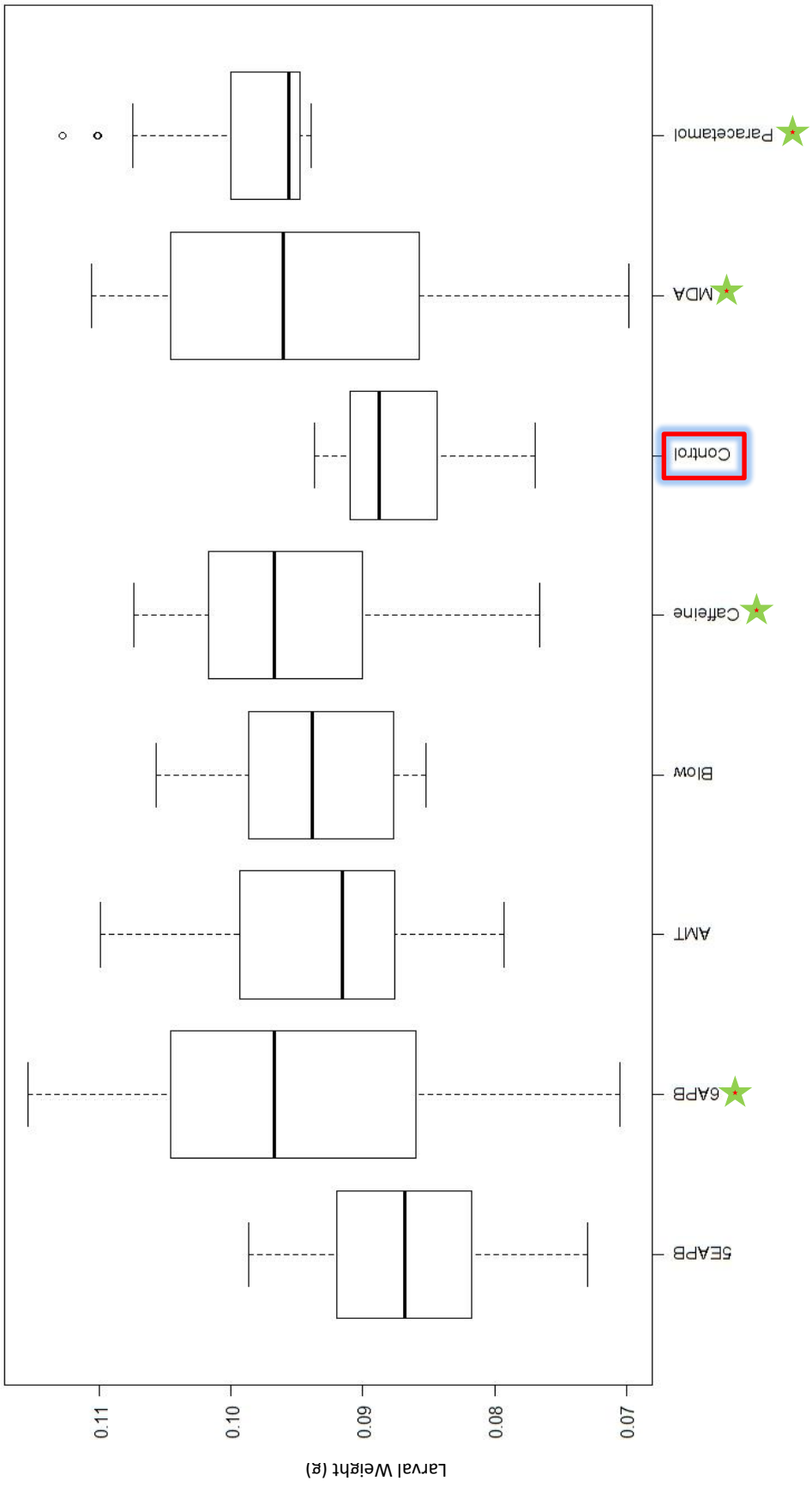


Figure 109: Box plot showing larval weight for day 8 across all samples (n=40).

Table 16: The minimum, maximum and mean lengths and weights (including standard deviation) on day 8 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
5EAPB	Length (mm)	13.1	15.577	14.6645	0.7259	4.95
	Weight (mg)	72.9	98.7	86.5	7	8.09
6APB	Length (mm)	15.55	17.13	16.4655	0.4978	3.02
	Weight (mg)	70.5	115.4	95.9	11.9	12.41
AMT	Length (mm)	14.67	18.12	16.883	0.896	5.31
	Weight (mg)	79.3	109.9	93.4	8.8	9.42
Blow	Length (mm)	16.031	17.62	17.0725	0.3811	2.23
	Weight (mg)	85.2	105.7	93.7	6.3	6.72
Caffeine	Length (mm)	15.5	17.64	16.1665	0.724	4.48
	Weight (mg)	76.6	107.4	95.1	8.6	9.04
Control	Length (mm)	15.35	17.82	16.5415	0.8037	4.86
	Weight (mg)	76.9	93.7	87.3	4.8	5.50
Paracetamol	Length (mm)	16.43	17.364	16.828	0.3094	1.84
	Weight (mg)	93.9	112.8	98.2	5.1	5.19
MDA	Length (mm)	15.554	17.136	16.4655	0.4945	3.00
	Weight (mg)	69.8	110.6	95.5	11.4	11.94

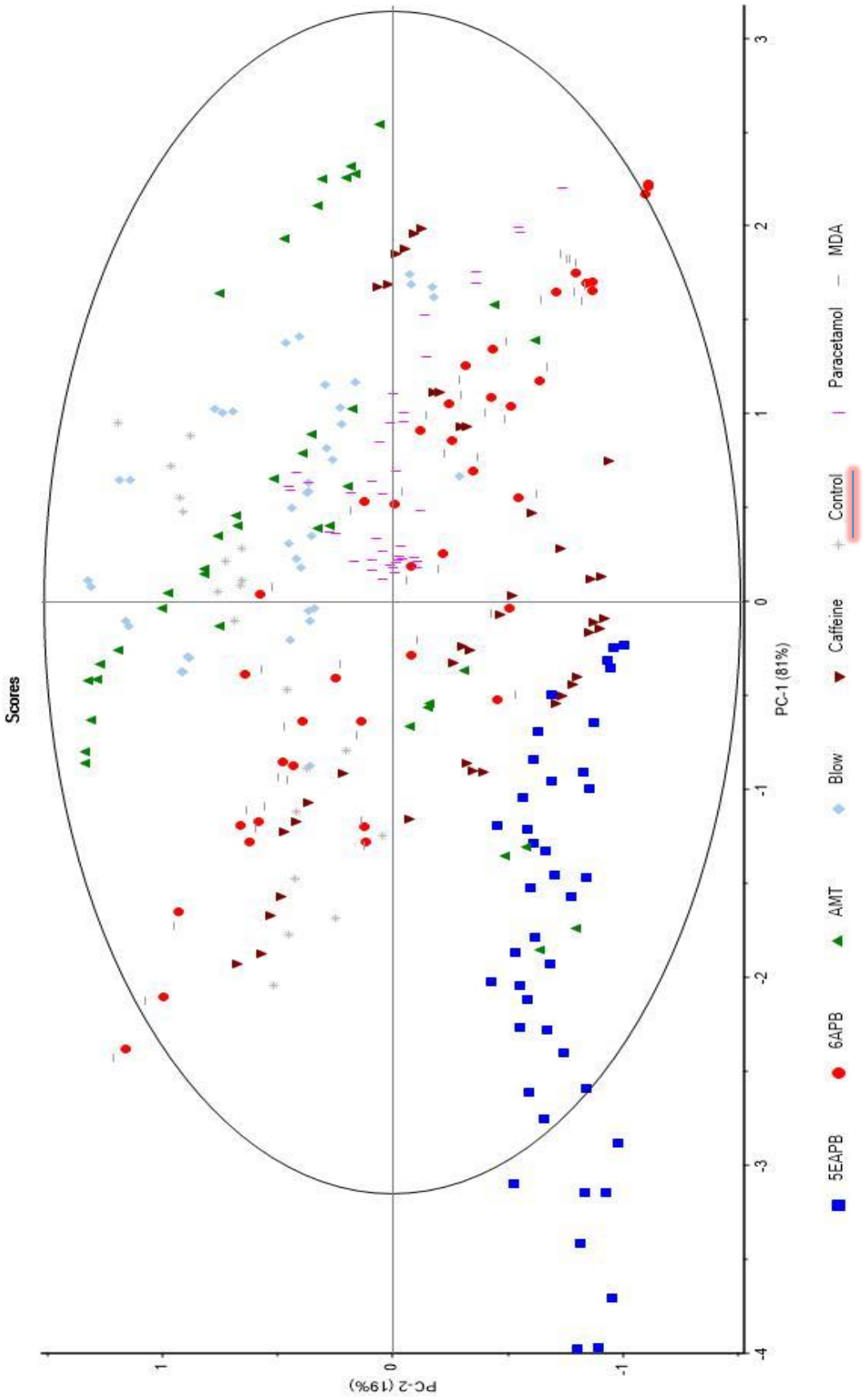


Figure 110: PCA plot showing all drug additions on day 8.

Day 8- Discussion

As hypothesized, samples showing measurement shrinkage during day 7, completed pupariation. The remaining drug diets are also now showing a reduction in size indicative of shrinkage prior to pupariation. Paracetamol however, is showing an increase in weight in comparison with the control diet. Development for this drug appears to have been considerably delayed.

Day 9

This sampling point saw the pupariation of a large number of the remaining drug diet samples. 5EAPB, Blow, 6APB, Caffeine, AMT and MDA all pupariated as expected due to their earlier development pattern. The remaining drug diets at day 9 only include the control and paracetamol populations.

Larval Length

Paracetamol larvae still appear larger than the control; lengths range from 13.7 mm to 17.66 mm, the control population has a mean at this time point of 15.1265 mm.

A mixed effects model considers these two diets significantly different ($F=23.116$, $P<0.0001$).

Larval Weight

Paracetamol also appears heavier at this time point. Weights ranged from 0.0782 g to 0.0913 g with the control having a mean weight of 0.0858g.

A mixed effects model still agreed a significant difference is shown between the two remaining populations ($F=12.477$, $P=0.0011$). Data set was normally distributed and uniform in variance.

Principle component analysis was performed and PC 1 and PC 2 were plotted showing 100% of the sample variance. They did show some overlap in values but differences were still clearly visible.

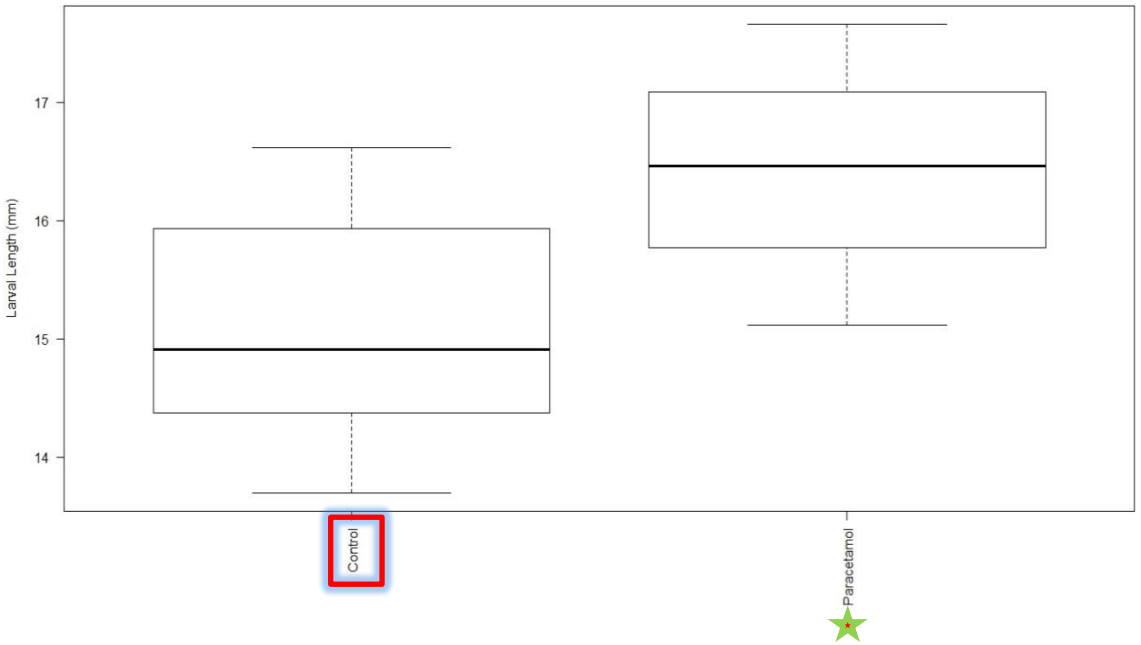


Figure 111: Box plot showing larval length for day 9 across all samples (n=40).

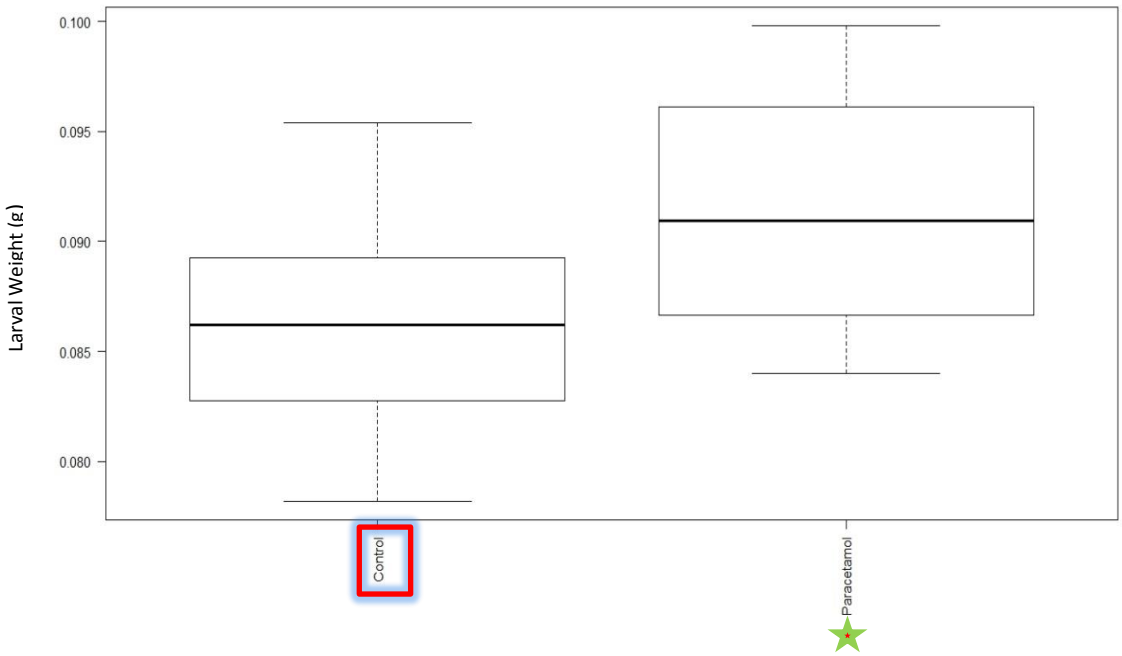


Figure 112: Box plot showing larval weight for day 9 across all samples (n=40).

Table 17: The minimum, maximum and mean lengths and weights (including standard deviation) on day 9 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
Control	Length (mm)	13.7	16.62	15.1265	0.9373	6.20
	Weight (mg)	78.2	95.4	85.8	4.6	5.36
Paracetamol	Length (mm)	15.12	17.66	14.415	0.7469	5.18
	Weight (mg)	84	99.8	91.3	5.2	5.70

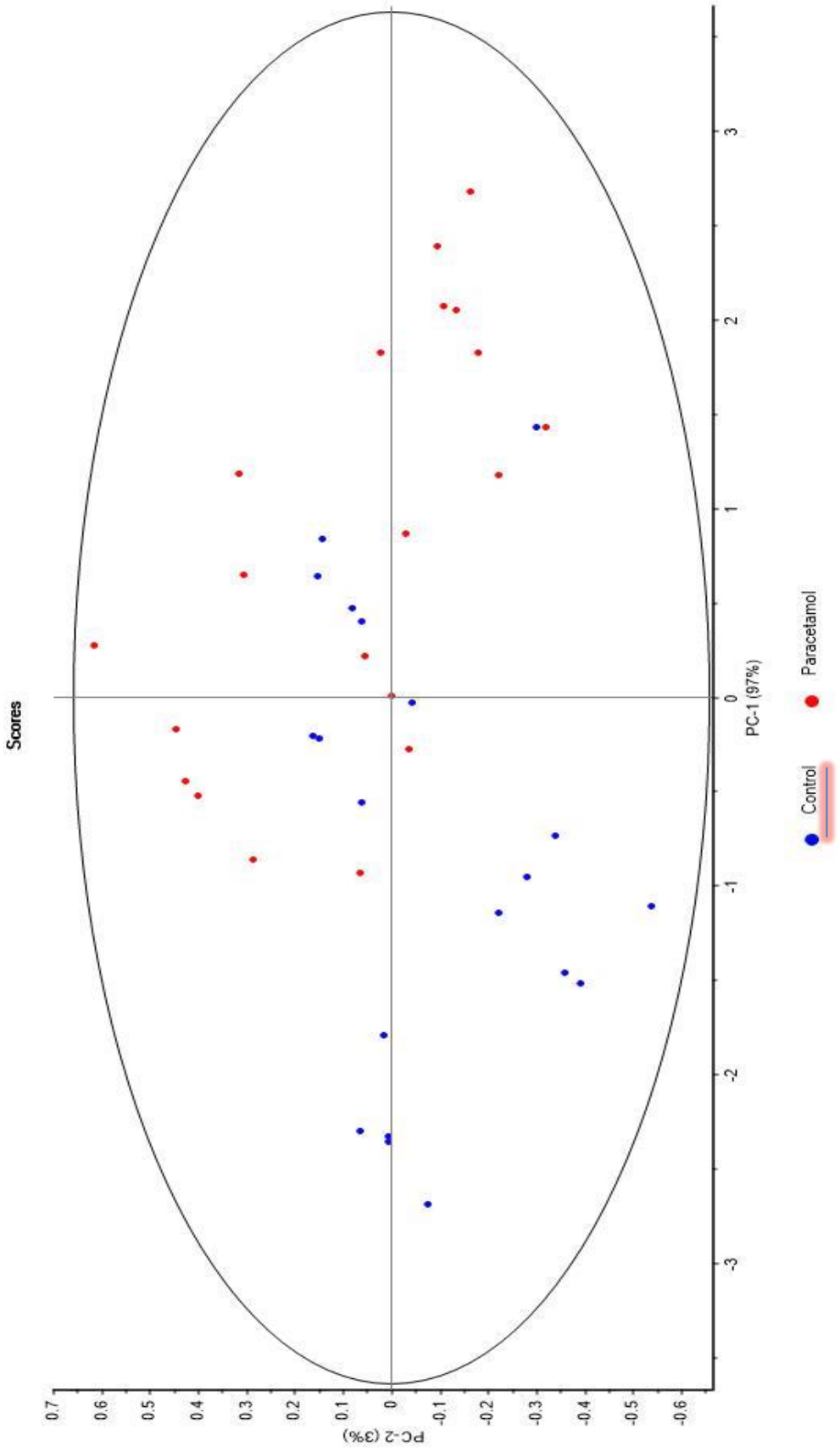


Figure 113: PCA plot showing all drug additions on day 9.

Day 9 – Discussion

The only remaining samples are now the control population and Paracetamol, which has continued along a pattern of delayed development. All other samples had pupariated prior to the sampling time point and the effects seen with the addition of these drugs to the larval diets must be considered for accurate post mortem interval estimation.

Day 10

By this sampling point, the control population had pupariated, leaving Paracetamol to continue development alone. For the concentrations used within this research, it can be shown that larval development was not only impeded in the measurements that were reached but also delayed in the time taken to achieve pupariation. At this stage, paracetamol had a mean length of 14.8365 mm, which is a slight increase compared with measurements from day 9. Larval weight has a mean of 0.0755 g, which is a decrease, compared with day 9, perhaps suggestive that development is plateauing. A scatter plot is shown in Figure 116 plotting the measurements taken at this time point, data is still shown to be positively correlated.

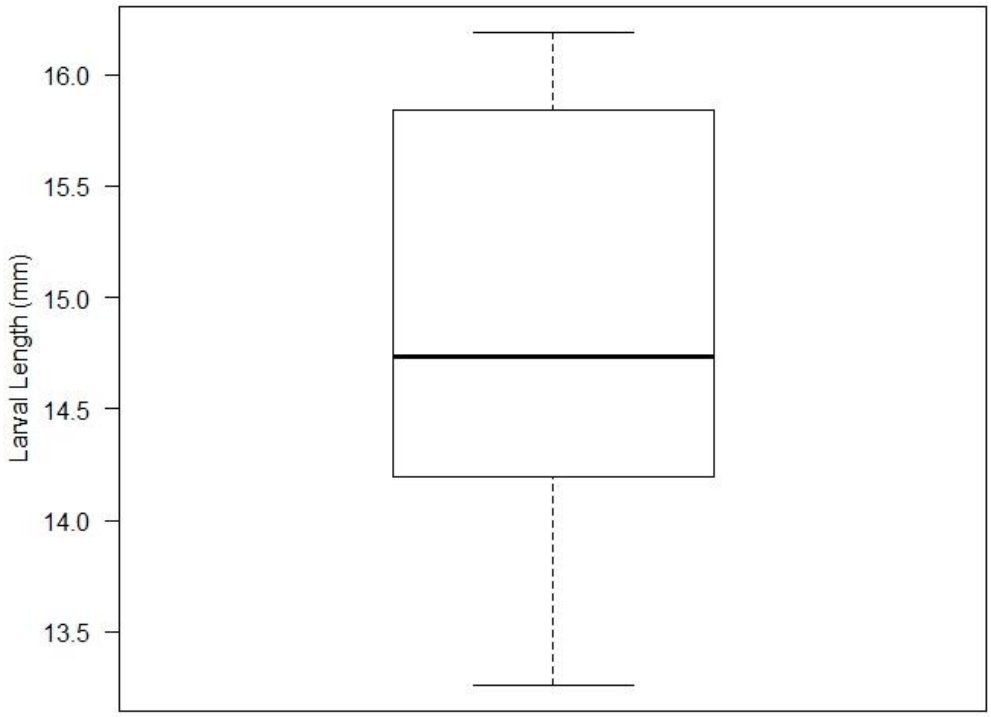


Figure 114: Box plot showing larval length for day 10 Paracetamol (n=40).

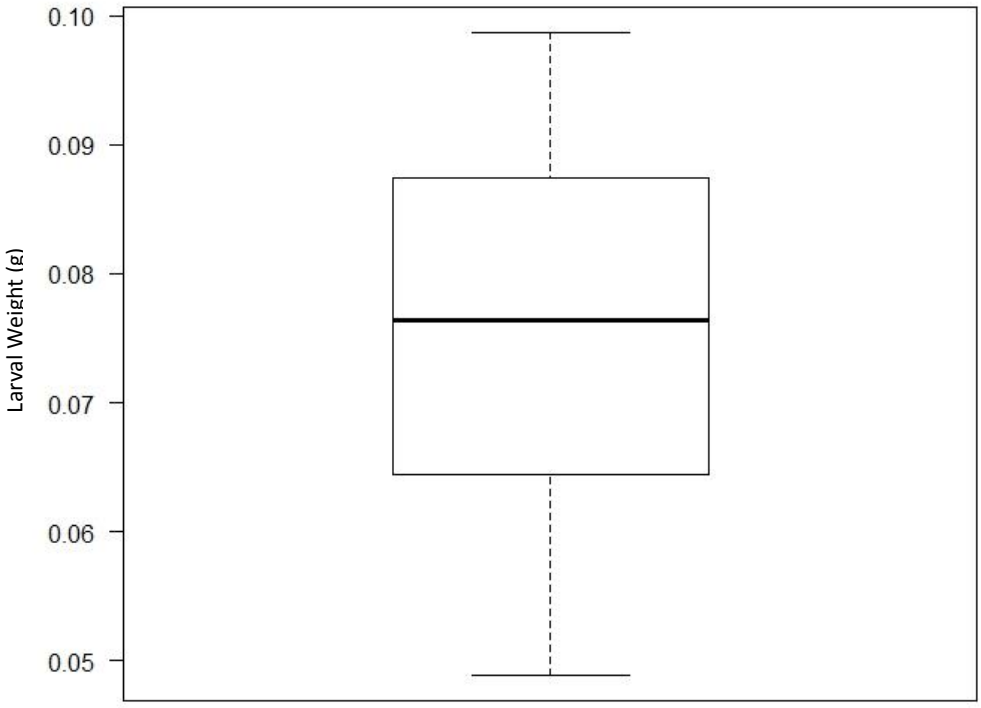


Figure 115: Box plot showing larval weight for day 10 for Paracetamol (n=40).

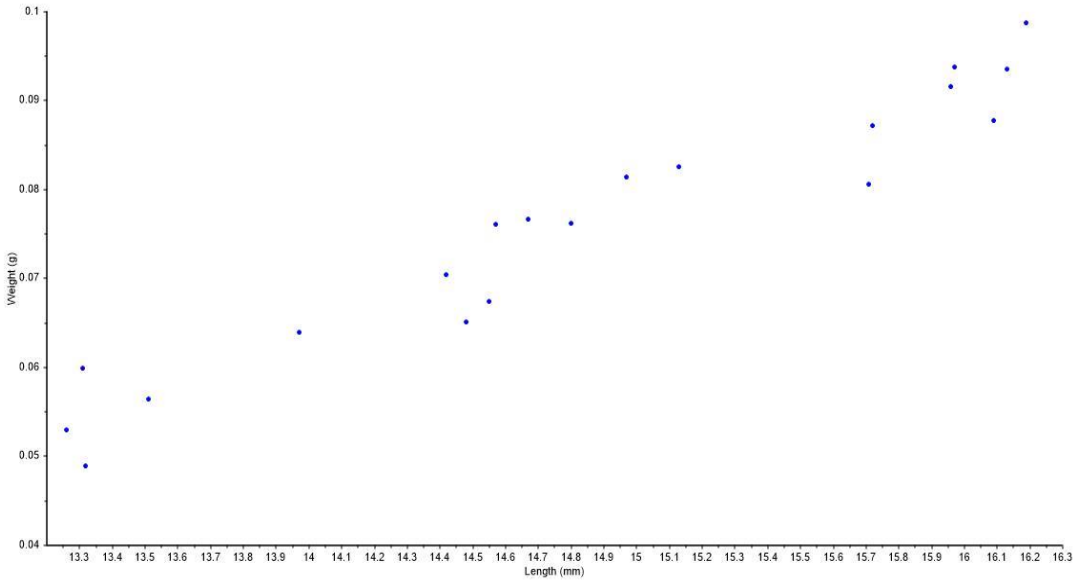


Figure 116: Scatter plot showing Paracetamol measurements on day 10.

Table 18: The minimum, maximum and mean lengths and weights (including standard deviation) on day 10 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
Paracetamol	Length (mm)	13.26	16.19	14.8365	1.0099	6.81
	Weight (mg)	48.9	98.7	75.5	14.5	19.21

Day 11

Larval Length

Larval length now has a mean of 14.072 mm, which has decreased since the previous sampling day.

Larval Weight

Larval weight is now 0.0734 g, also showing a slight decrease from day 10.

By the next sampling period, Paracetamol had finally pupariated; this was 48 hours after the control population were shown to pupate. This could cause significant misinterpretation of minimum post mortem interval.

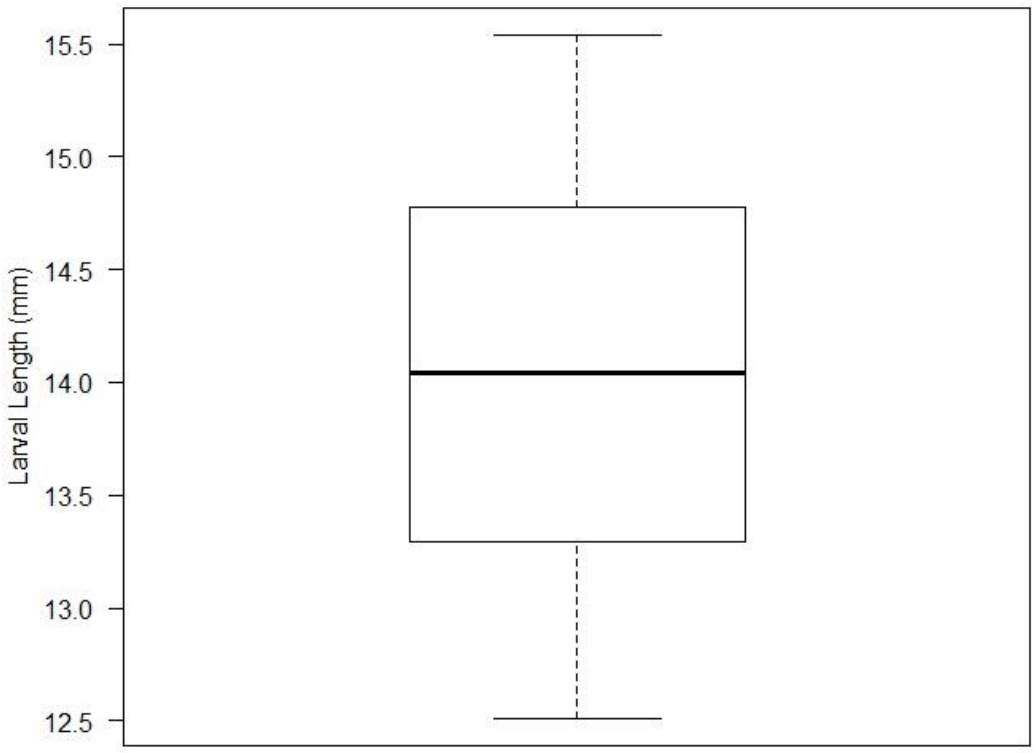


Figure 117: Box plot showing larval length for day 11 Paracetamol (n=40).

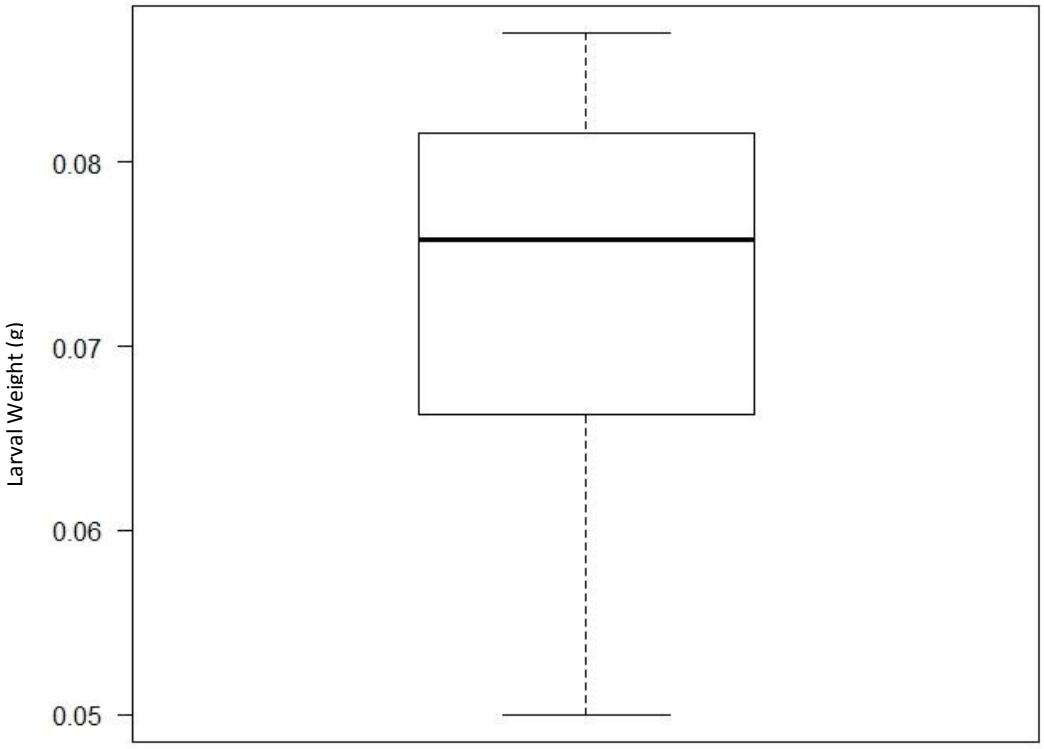


Figure 118: Box plot showing larval weight for day 11 Paracetamol (n=40).

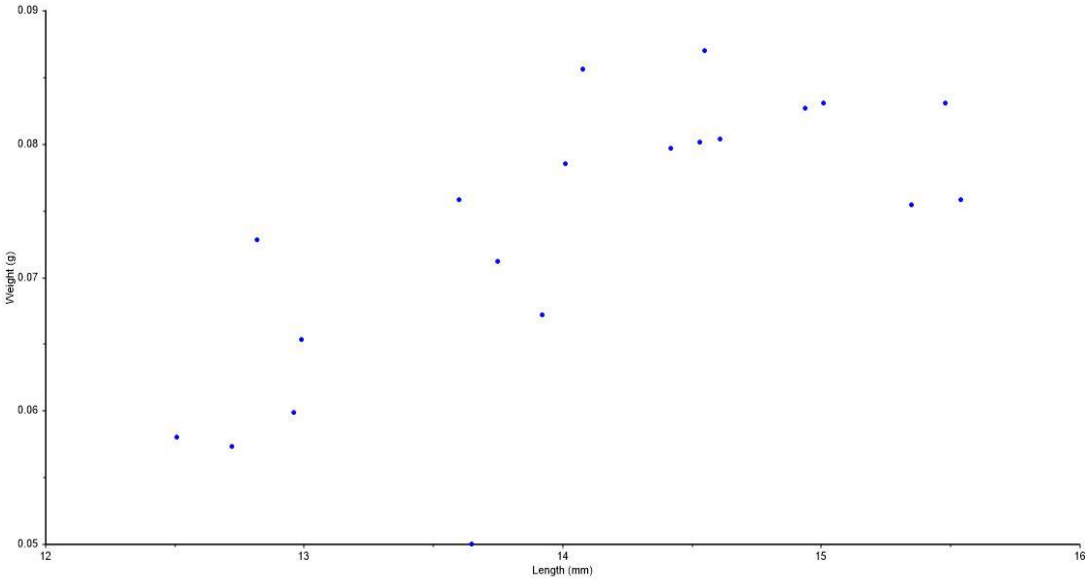


Figure 119: Scatter plot showing Paracetamol measurements on day 11.

Table 19: The minimum, maximum and mean lengths and weights (including standard deviation) on day 11 (n=40).

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
Paracetamol	Length (mm)	12.51	15.54	14.072	0.09467	0.67
	Weight (mg)	50	87	73.4	10.6	14.44

PCA changes across the data set

A PCA plot concerning 100% of the data variance is shown in Figure 120 with PC 1 and PC 2. Included is data from two selected drugs and the control across 11 days development. The drugs selected are 5EAPB and Paracetamol, these appear to have opposing effects on the larval population and it is of interest to determine if each day will cluster regardless of the drug treatment or if different days may show data similarities suggesting potential inaccuracies when estimating PMI.

An overview of all days is shown in Figure 120 and although some clustering is visible, it is unclear with this large amount of data. Days 1-3, 4-6 and 7-11 will therefore be shown in individual PCA plots in figures Figure 121 to Figure 123.

Day 1 samples appear to cluster together, which is unsurprising given the short amount of exposure to the drug diets at this point. Day 2 appears to show three individual clusters with 5EAPB quite widely spread. Control appears to sit in between these two datasets and Paracetamol is moving towards the cluster of day 1 results. In day 3, 5EAPB and Control share some overlap but some grouping is visible. Paracetamol however is clustering more

so with day 2 samples, apparently showing more similarity to individuals of 48 hours old opposed to 72 hours old.

Figure 122 shows data from day 4-6. At this stage, it becomes apparent that diets are no longer clustering with their sampling day. Day 4 Paracetamol sits alone on the left-hand side of the plot while control sits more centrally, again mostly isolated. Day 4 5EAPB appears to cluster centrally alongside samples from day 5 and day 6. Overlap shows areas of misinterpretation.

Figure 123 shows day 7 to day 11. Clustering is certainly visible; but at this stage without colour coding the sample groups, it would be hard to identify these.

The conclusion being that samples do not tend to cluster within each individual sampling day (Clustering would show similarities between the days). This leaves data interpretation of developmental measurements open to large over and under estimations for minimum post mortem interval.

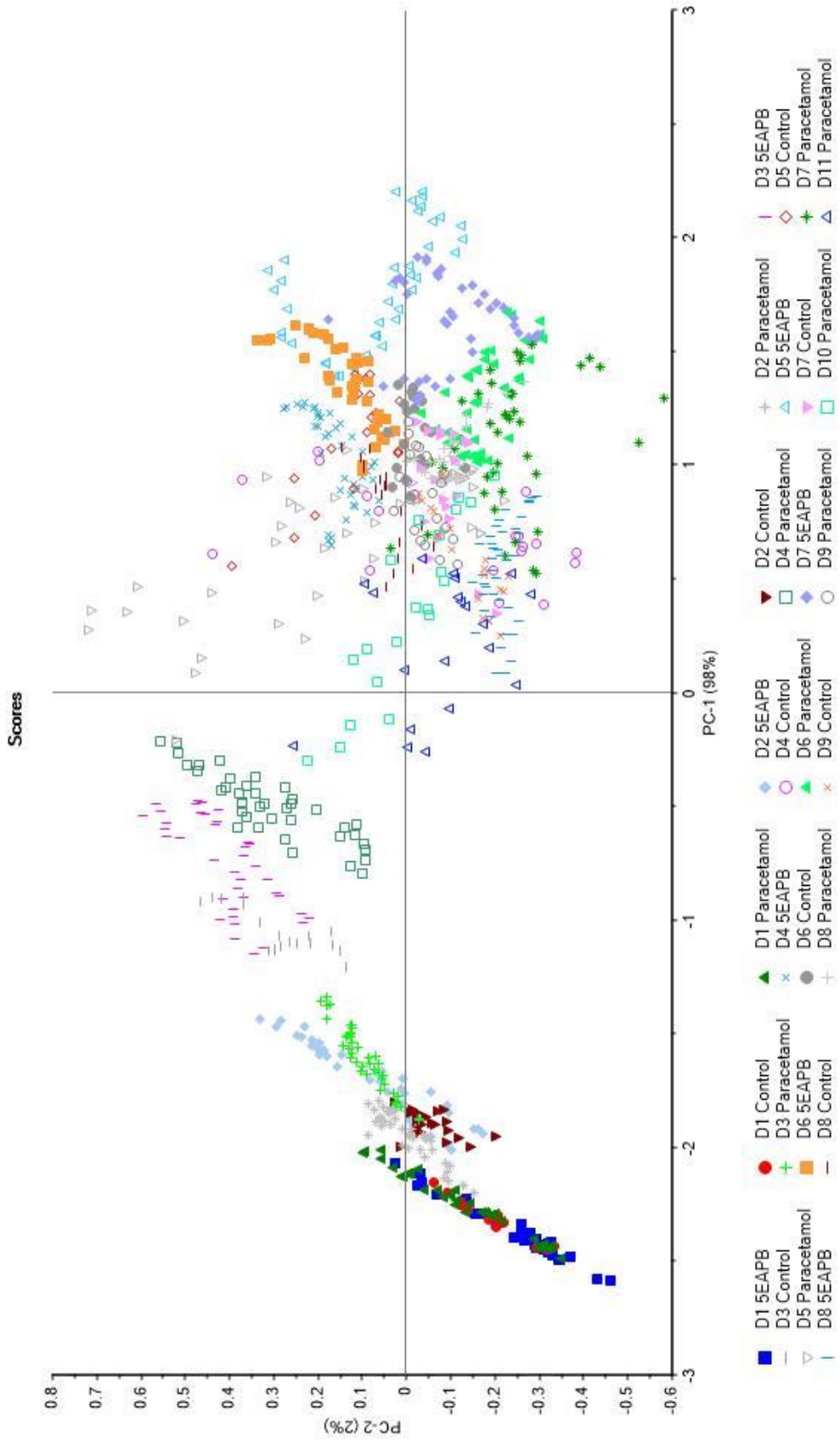


Figure 120: PCA plot showing selected drug diets across days 1-11.

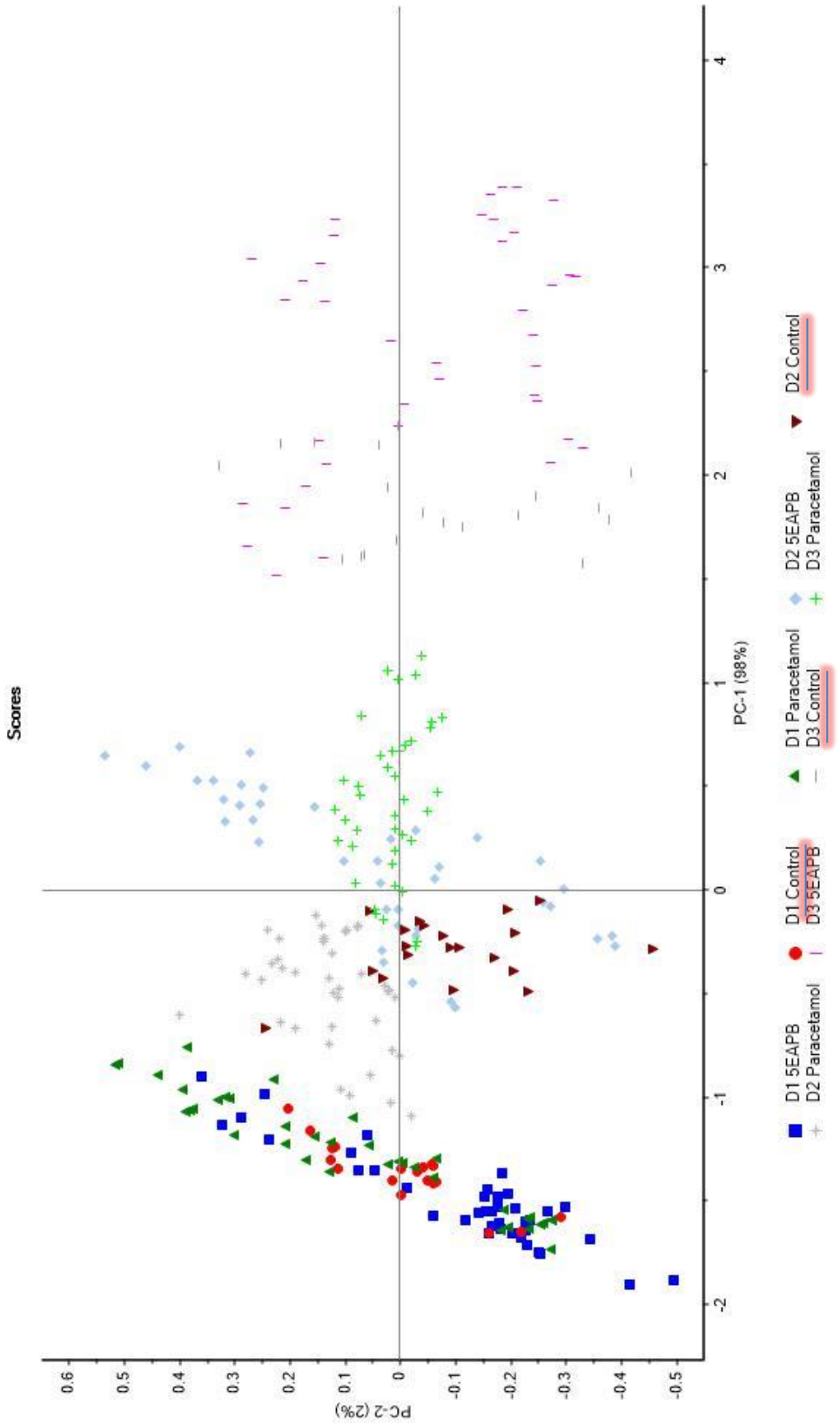


Figure 121: PCA plot showing selected drug diets across days 1-3.

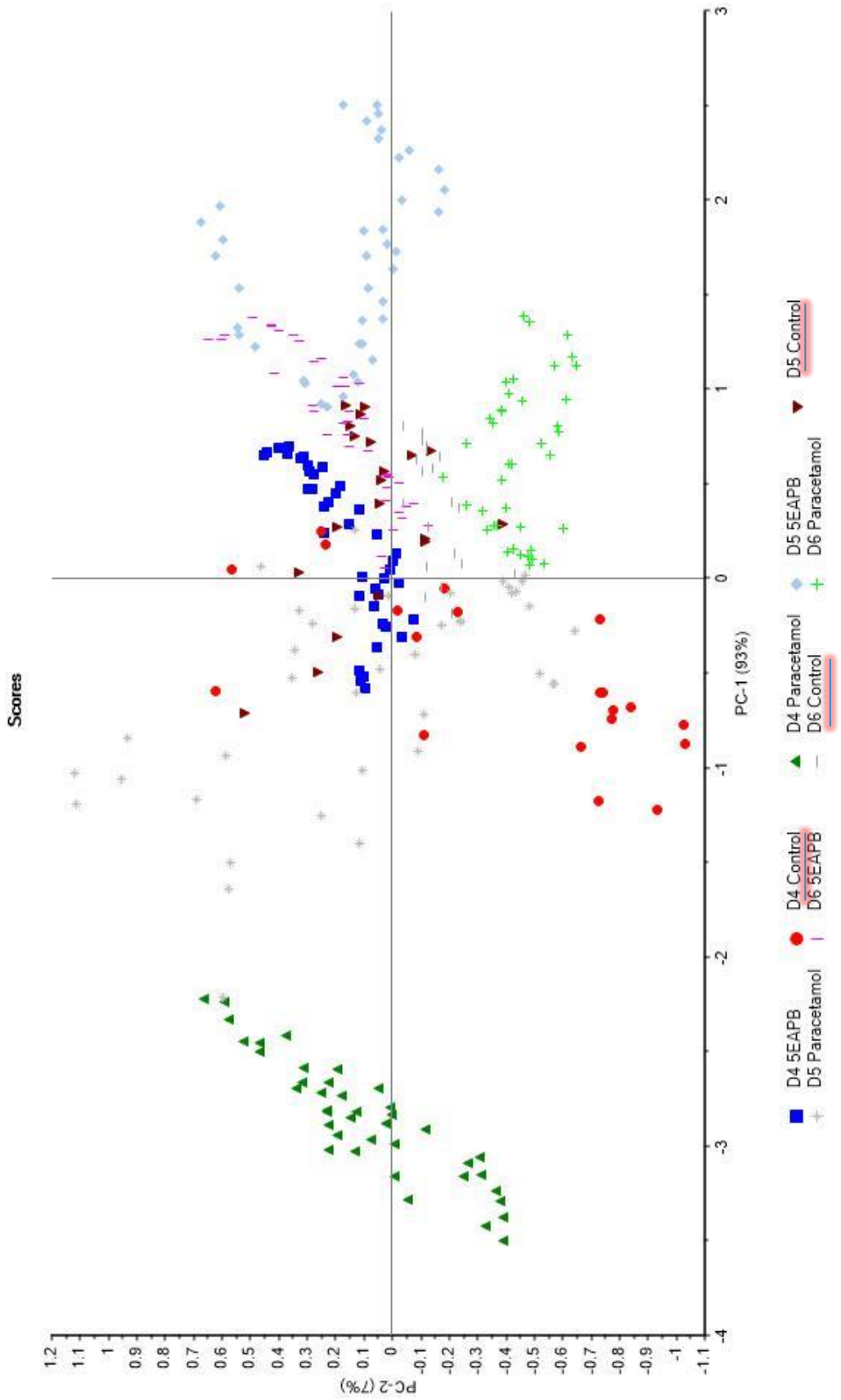


Figure 122: PCA plot showing selected drug diets across days 4-6.

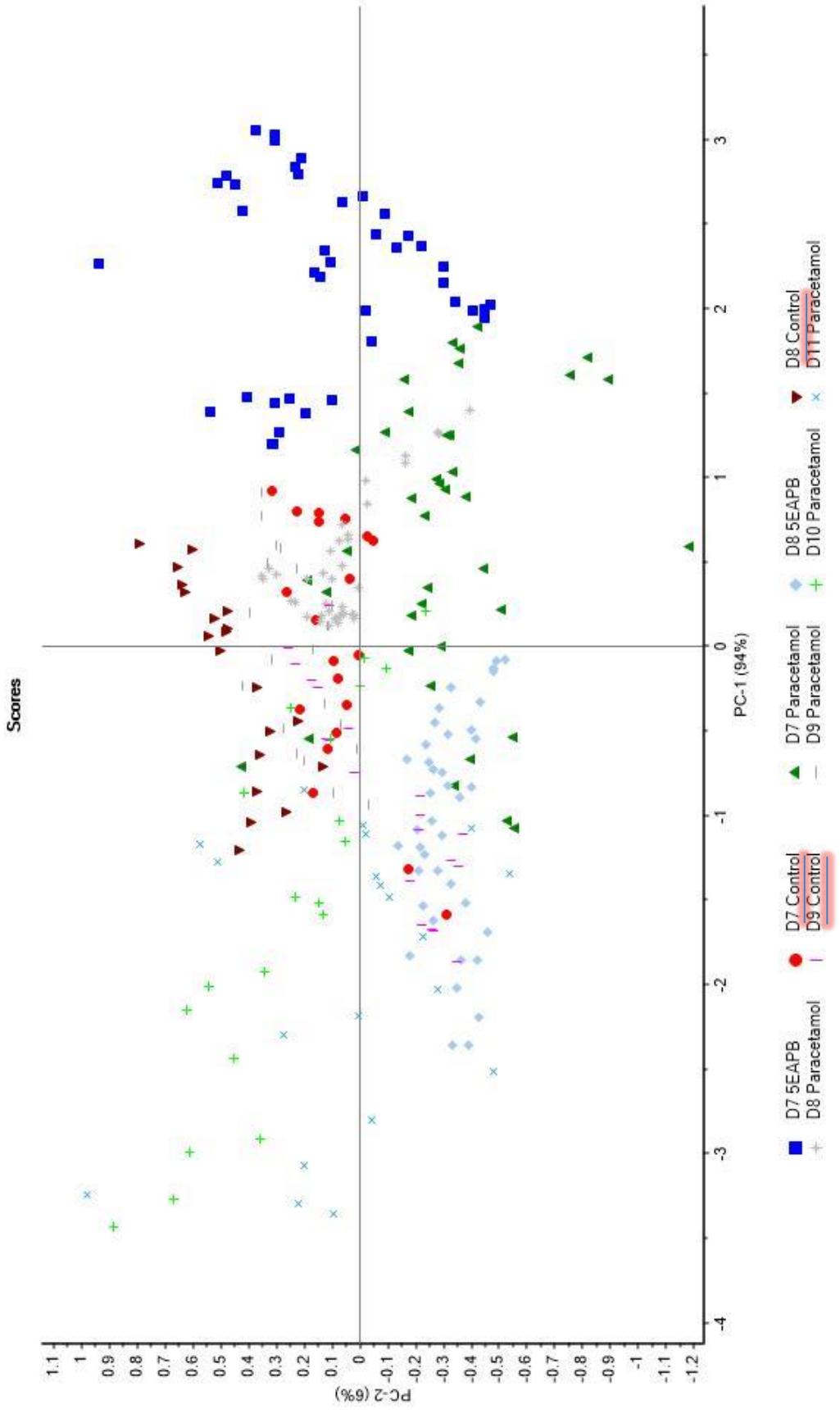


Figure 123: PCA plot showing selected drug diets across days 7-11.

5.2.3.1 Overall Development Discussion:

This study suggests that when Novel Psychoactive Substances have been ingested prior to death and are therefore present in a corpse, there is the potential to cause significant bias when estimating minimum PMI. This has been previously shown in relation to chemical compounds in a number of studies; for a review, the reader is referred to Chapter 1.2.

No difference was initially seen at day 1 due to insufficient time to allow effects of the drugs upon development at an early sampling point, this was also shown in other research [150].

An increase in development has been observed due to the exposure to drugs such as heroin [90], methamphetamine [154], codeine [132], [306] , cocaine [105], [128], [150] and paracetamol [304]. Substances have also been found to decrease the rate of larval development, such as diazepam, morphine and methadone [102], [134], [147]. Development was shown to increase for all drugs in this study with the exception of Paracetamol, which delayed development quite significantly.

Drugs and toxins are known to affect the behaviour of larvae and this includes the manner in which they feed. One study focussed on the effects on Cocaine, showed that a larger dosage elevated the activity of the larval population, including rapid feeding and resulting in increased drug consumption [150]. The rate of development was then shown to increase through the larval stages before slowing when feeding is halted upon entering the post feeding stage. It is at this stage that the drug concentration within the individual larvae would drop as the rate of elimination and metabolism will exceed the rate of ingestion.

The data certainly does suggest that the organoleptic properties of the diet are influenced with the addition of drugs and toxins. This could potentially delay pupariation as larvae are known to feed until they reach an ideal weight, when they will then slow for pupariation (the plateau we see in developmental data) and food is eliminated from the gut [148]. Prior to this stage, larval weight appears to follow a sigmoid pattern. For most of the drugs shown within the scope of this thesis, pupariation however, is not delayed, with the exception of Paracetamol. This suggests that larvae overcome the issue with the drug presence, in some way. There have been previous studies that show limitations in relation to food (quality or quantity) and available space can result in smaller adult flies [307]. The sizes of the adult blowfly resulting from all drug diets did not appear to differ, although this was not measured in terms of weight, there was no physical indication of smaller adults, this suggests that although certain diets did appear to be less palatable, the larvae still reached the nutritional threshold to enable full unimpeded development.

This Paracetamol study suggests that the drug negatively influences larval development. A previous study contradicts this suggesting that Paracetamol slightly affected larval development during day 2-4 only, where growth was accelerated. A range of concentrations were used in the study, 100 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg but no differences was found to be dose dependant [304]. Potential reasoning behind these differences could concern feeding substrate, colony differences, mass size or sampling protocol including photoperiod and temperatures. The data shown in this thesis utilises a researched artificial diet as opposed to homogenized pork liver. Preliminary studies shown in this thesis suggested that larvae provided with a drug within a homogenised meat substrate would show different developmental changes than

expected, due to the uneven distribution of drugs. The unpalatability caused by the drug appeared to encourage larvae to move on to another area of the diet where the drug was not present. Although the selected species was the same, the origin of the species in the research is unknown. One major difference seen between the two experimental procedures was mass size, the research shown in this thesis used larger mass sizes; larger spaces and sufficient food was provided to deter competition. Turner [304]used a mass of 20 larvae. This would affect digestion of the diet and liquefaction. This warrants further investigation and shows the importance of a defined protocol for entomotoxicological studies.

5.2.3.1.1 Adulterants

Paracetamol was shown to significantly delay the development of blowfly larvae, particularly at high concentrations. Benzocaine and caffeine were shown to accelerate development and initiate early pupariation but also produced larval measurements during all instars that were significantly different and would have introduced PMI estimation errors.

5.2.4 Does dosage make a difference to the developmental changes observed?

Two different dosages were selected for each of the drugs highlighted within this thesis, drug concentrations were selected to provide a high and low dosage. Dosages chosen within this research were considered to be representative of those found within the system following a fatal drug overdose, see Table 8 for concentrations used.

It is hypothesized that the drugs shown to accelerate development will accelerate growth further with higher dosages. A point will be reached where the dosage may kill the blowfly larvae. For drugs shown to impede development, it is suggested that a higher dosage will impede development further, although all substances have been tested at two doses only.

Four drugs at their two selected dosages and the control will be discussed in this chapter. The drugs, which have been selected, are AMT, Blow, Caffeine and Paracetamol. Reasoning for this selection is due to the variety of developmental differences seen within the initial analysis. Data is shown for alternate days initiating with day two to enable sufficient time for ingestion of the compounds.

Day 2

Larval Length

Lengths range from 5.43 mm to 8.39 mm within this selection of compounds on day 2 of sampling. The control has a mean larval length of 6.811 mm. AMT previously showed no significant difference when compared to the control. Comparing each dosage individually

to the control also shows no significant differences. The higher dose does however show more difference than the lower dose with these two dosages shown to be significantly different to each other ($P<0.01$). The lower dosage ranges between 6.21 mm and 7.104 mm while the higher dosage ranges between 5.75 mm and 7.17 mm, the higher dosage appears to be affecting development by slowing it down, more so than the lower dosage. AMT has been shown in the original combined analysis to initially slow development and then suddenly accelerate between sampling days 4 and 5. This hampering of development could be a direct result of the drug or alternatively, could be resistance from the larval population to ingest the compound containing diet and therefore slowing their own development. This has been investigated in Chapter 4, however, different dosages were not considered. It can be hypothesized that the lower dosage is more palatable for the larvae compared with the higher dose. Original analysis of Blow showed it too resulted in larvae larger than the control, this trend appears to continue with the dose separation ($P<0.01$). The two dosages are not shown to be statistically different although a difference in data range can be seen in the bar chart in Figure 124. The lower dose ranges between 6.11 mm and 8.19 mm and higher dose between 6.75 mm and 8.17 mm, the difference is small but the lower dose does result in lower measurements. Caffeine was shown previously to be significantly different to the control at this sampling point, analysis of the doses separately suggests the higher length values to be caused by the higher dosage, the higher dose is significantly different to the control ($P<0.01$), whereas the lower dose is more similar to the control population. Results for the separate analysis of Paracetamol show that the results do not appear that different; the lower dose can visually be seen to be causing larger lengths than the higher but with a number of lower outliers.

Larval Weight

AMT had been shown previously to be significantly smaller than the control population when considering larval weight. When the doses are considered separately, they are both shown to be significantly different to the control and also from each other ($P<0.001$). The lower dosage resulted in larger weights, possibly showing a larger amount of the diet had been consumed. The higher dose gave lower larval weights perhaps suggesting that the population is impeded by the unpalatability of the diet. When Blow is analysed separately they are both considered significantly different to the control population but not from each other. Any effect caused by the different dosages appears to be minimal. It can be seen from the weight ranges that the higher weights are achieved from the higher dose, it is apparent that the presence of a high dose does not have the same result as seen previously. Caffeine was not considered different to the control when analysing data as a whole, no significant differences were seen when analysing the doses separately. Paracetamol was considered to weigh less than the control previously, both dosages also agree with this ($P<0.001$) but no differences are shown between the dosages.

Principle component analysis was carried out utilising PC 1 and PC 2, which together showed 100% of the data variance. Different dosages do appear to cluster separately apart from Paracetamol and this is due to the measurement differences already shown.

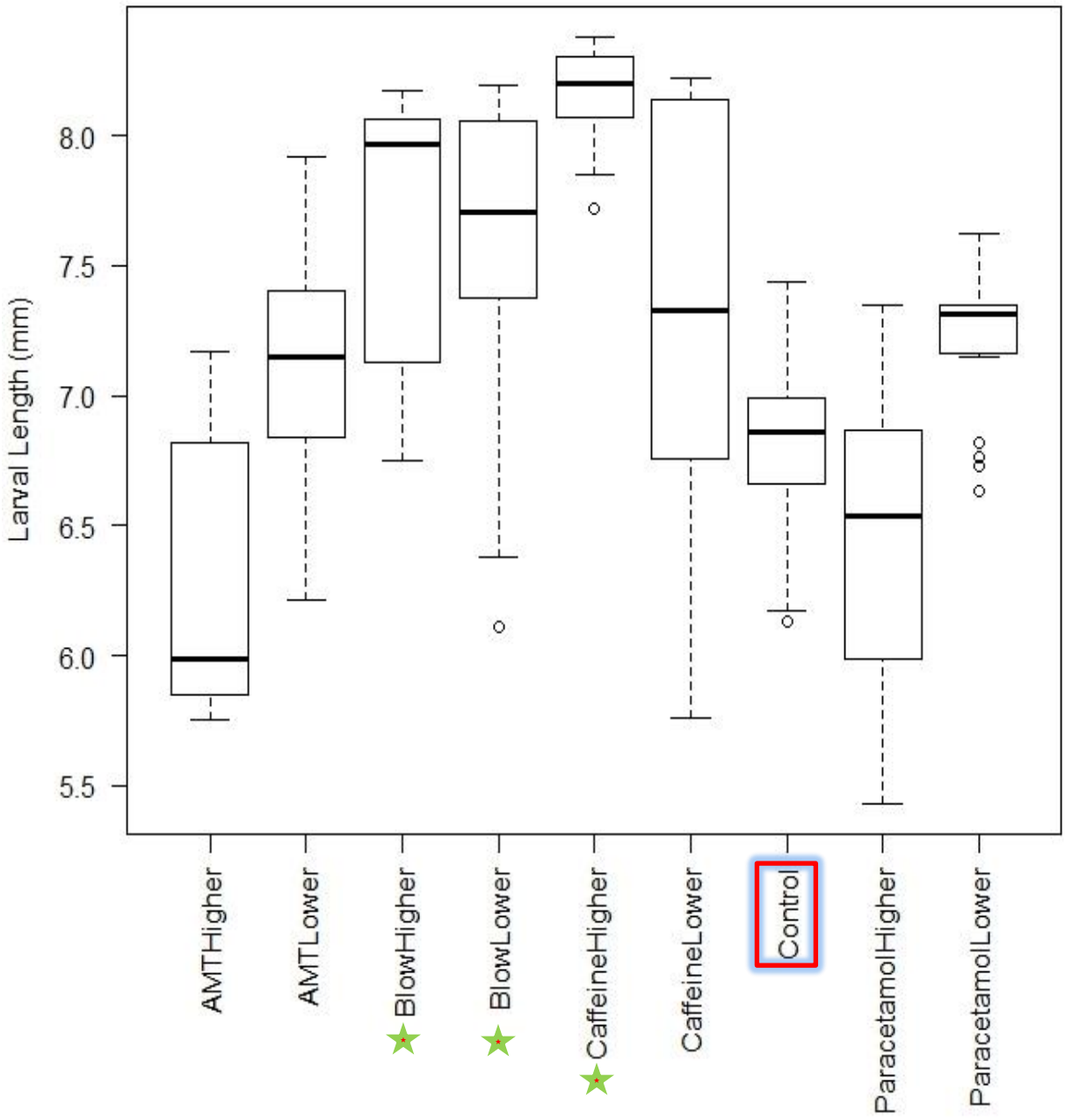


Figure 124: Box plot showing larval length for day 2 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

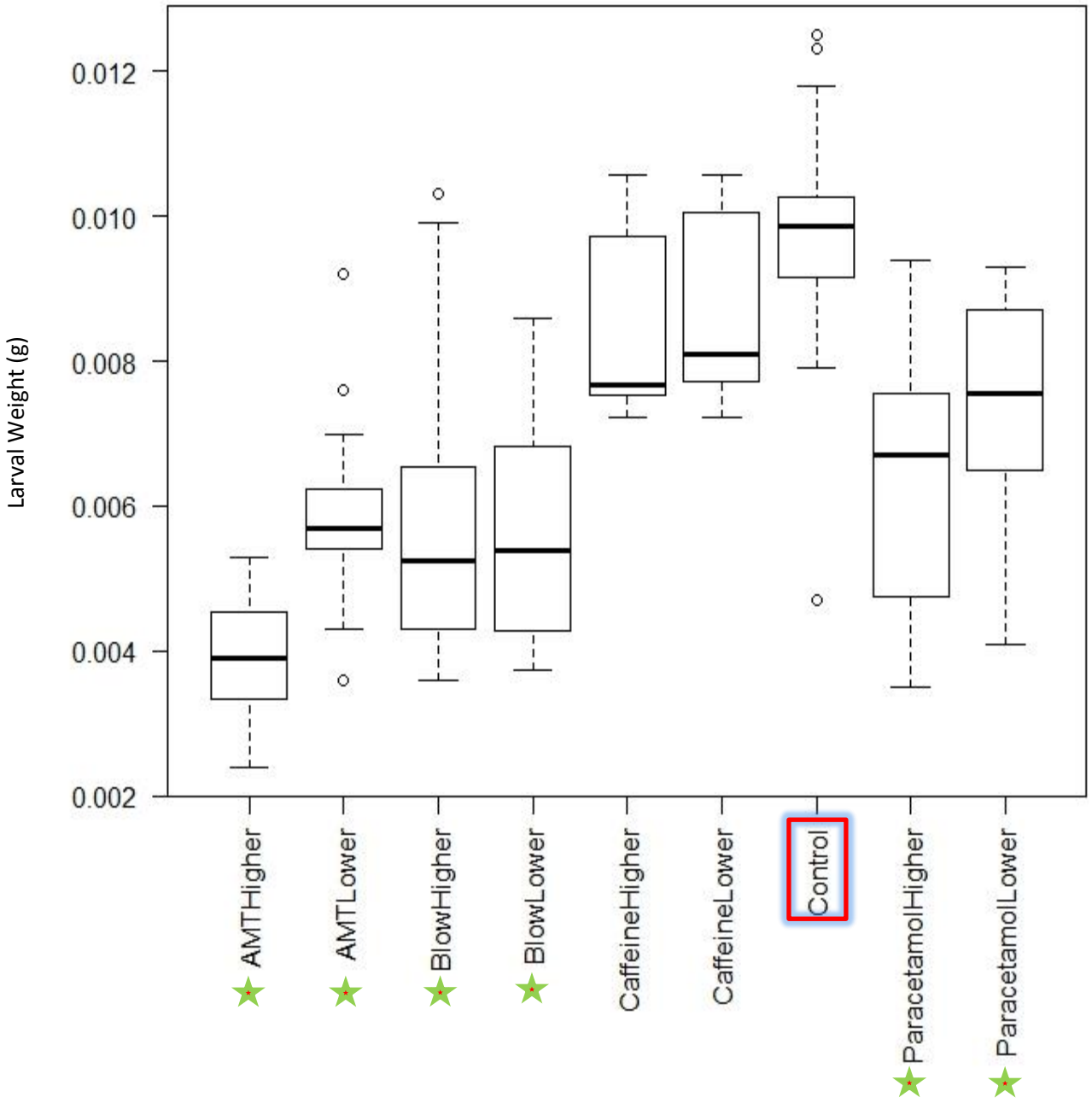


Figure 125: Box plot showing larval weight for day 2 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

Table 20: The minimum, maximum and mean lengths and weights (including standard deviation) on day 2 of selected drugs and dosages. The reader is referred to Table 8 for concentrations.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
AMT Lower	*Length (mm)*	6.21	7.92	7.104	0.47	6.67
	Weight (mg)	3.6	9.2	6	1.40	23.33
AMT Higher	*Length (mm)*	5.75	7.17	6.2715	0.53	8.39
	Weight (mg)	2.4	5.3	3.9	0.80	20.51
Blow Lower	*Length (mm)*	6.11	8.19	7.543	0.63	8.40
	Weight (mg)	3.7	8.6	5.7	1.60	28.07
Blow Higher	*Length (mm)*	6.75	8.17	7.653	0.52	6.83
	Weight (mg)	3.6	10.3	5.9	2.10	35.59
Caffeine Lower	*Length (mm)*	5.76	8.22	7.329	0.79	10.77
	Weight (mg)	7.2	10.6	8.7	1.20	13.79
Caffeine Higher	*Length (mm)*	7.72	8.38	8.1575	0.19	2.28
	Weight (mg)	7.2	10.6	8.5	1.30	15.29
Control	*Length (mm)*	6.13	7.44	6.811	0.33	4.88
	Weight (mg)	4.7	12.5	9.7	1.70	17.53
Paracetamol Lower	*Length (mm)*	6.63	7.62	7.2275	0.28	3.87
	Weight (mg)	4.1	9.3	7.4	1.50	20.27
Paracetamol Higher	*Length (mm)*	5.43	7.35	6.4545	0.54	8.36
	Weight (mg)	3.5	9.4	6.2	1.70	27.42

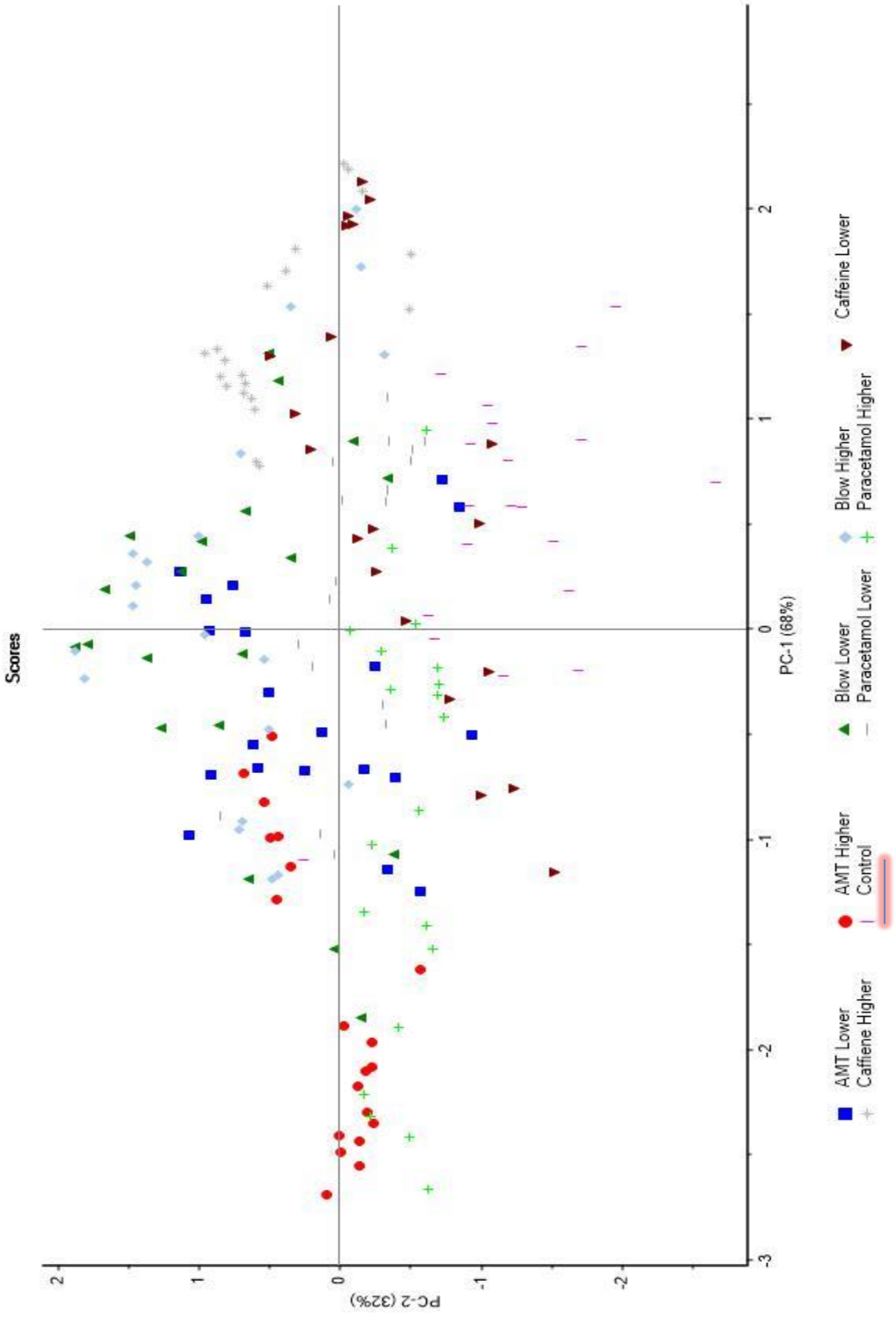


Figure 126: PCA plot showing selected drugs and dosages on day 2. The reader is referred to Table 8 for concentrations.

Day 2- Discussion

Differences are shown between dosages at day 2. These differences are concerned predominantly with larval length as opposed to weight. Caffeine and Paracetamol appear to agree with the initial hypothesis, a drug impeding development will impede further at a higher dosage and a drug accelerating development will accelerate further when the dose is higher. AMT and Blow appear to currently show apposing patterns, which will be analysed further as the larval lifecycle continues.

Day 4

Larval Length

AMT was shown not to be significantly different from the control sample at day 4 and separate analysis of the doses agreed. The range of measurements suggests that a higher dose impeded development more. An outlier is shown in the lower AMT dosage. Blow was also shown not to change with separate analysis of the dosages; again, the range showed that the higher dose impeded development more so than the lower dose. Caffeine had previously been shown as resulting in significantly larger larvae. Separate analysis of the data suggests that this difference is caused mainly by the higher dosage as the lower dose does no cause a significant difference. The higher dose appears to be accelerating the growth rate more ($P<0.001$). Separate analysis of Paracetamol does not alter the final result as both show to be significantly lower than the control. They are however; significantly different from each other also, the higher dose impeded development further.

Larval Weight

Prior analysis of AMT showed a significant decrease in weight in comparison to the control. Separate analysis shows both dosages also follow this pattern ($p<0.0001$), but do not appear that different from each other. The lower dose has a narrower range of data so perhaps the effect is less varied. Separate analysis of Blow agrees that larval weight is significantly reduced when compared to the control group. These doses also show significant differences between themselves ($P<0.001$), with the lower dose causing lower values and the higher dose increasing, especially considering the previous developmental measurements. Caffeine analysis again agreed with the initial analysis as significantly lower when separately analysed. A difference was found between the dosages ($P<0.01$) but they had comparable means. The lower dosage had measurements spread over a wider range, whereas the higher dosage appears to be comprised of a smaller range of measurements at the higher end of the range seen for the lower dosage. Paracetamol analysis did not alter with separation of the data into doses, both were considered significantly smaller than the control samples and no significant differences were shown between them.

Principle component analysis taking into account both length and weight, showed three distinct clusters which comprised of both doses of Paracetamol in one, Control in another and the Caffeine, Blow and AMT doses in another. Analysis of this larger cluster shows some further clustering, but with some overlap between dosages, where data shows similarities.

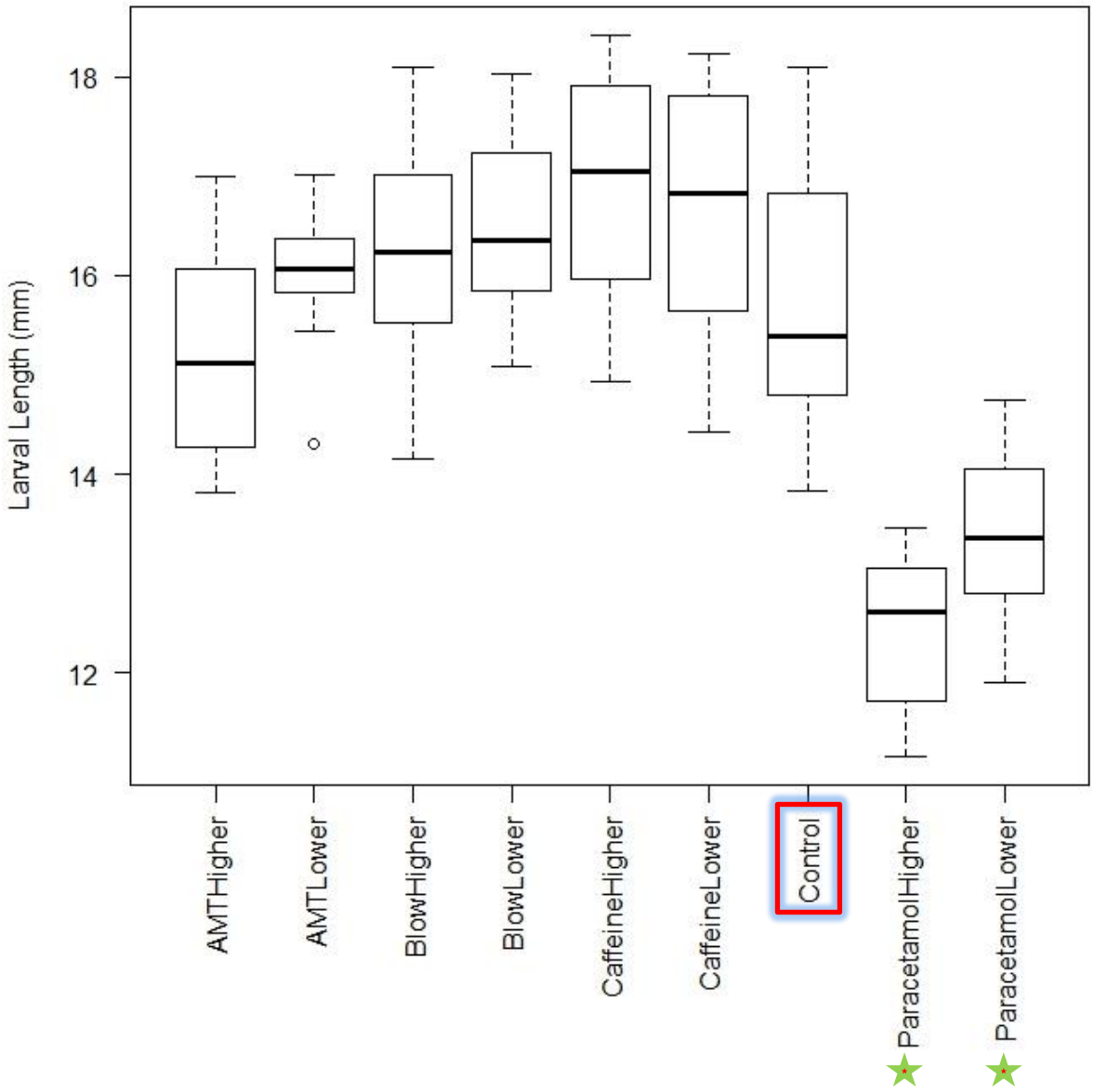


Figure 127: Box plot showing larval length for day 4 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

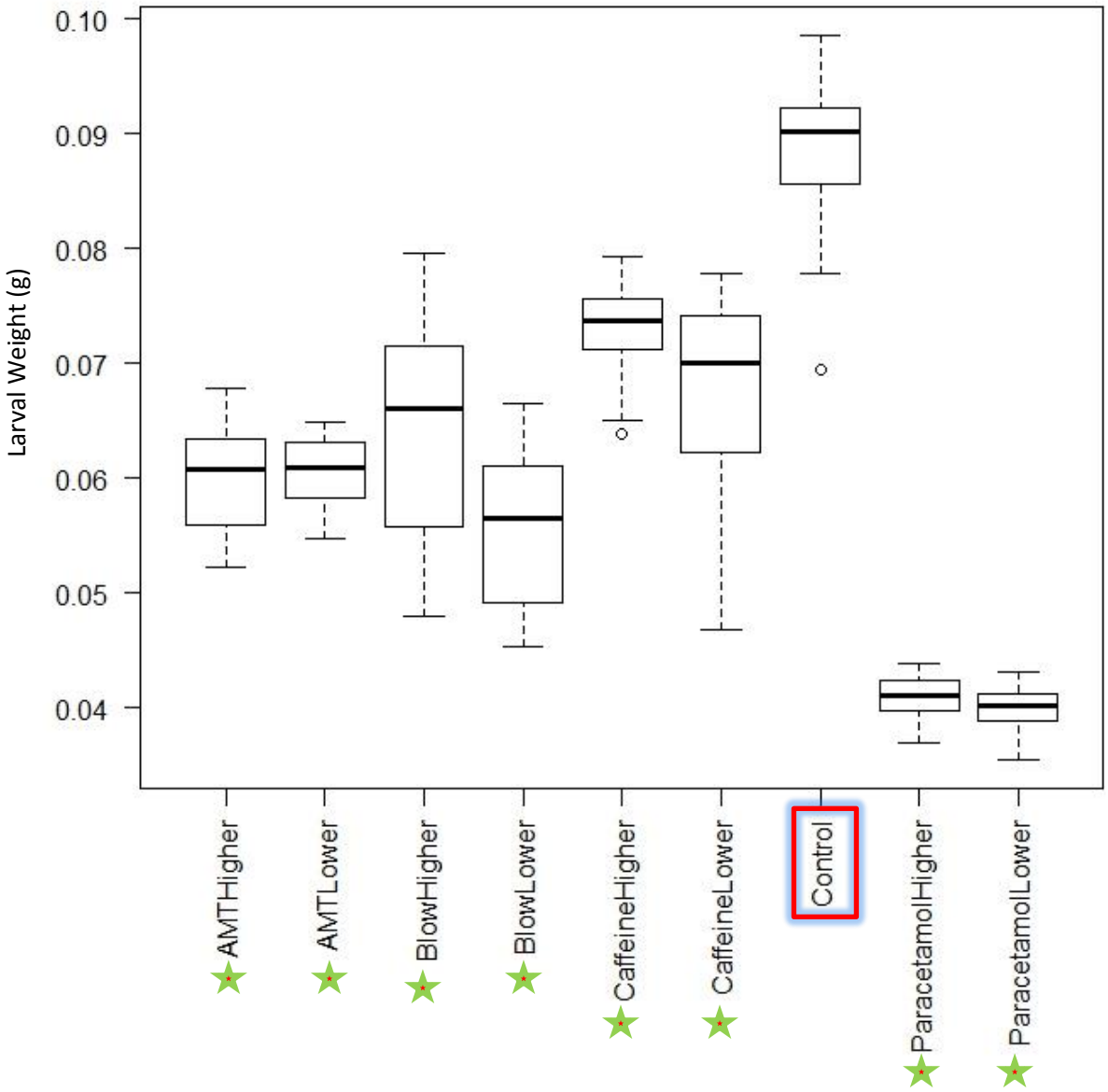


Figure 128: Box plot showing larval weight for day 4 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

Table 21: The minimum, maximum and mean lengths and weights (including standard deviation) on day 4 of selected drugs and dosages. The reader is referred to Table 8 for concentrations.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
AMT Lower	*Length (mm)*	14.31	17.01	16.048	0.58	3.59
	Weight (mg)	54.8	64.9	60.4	3.30	5.46
AMT Higher	*Length (mm)*	13.81	16.99	15.176	0.99	6.49
	Weight (mg)	52.2	67.8	59.9	4.90	8.18
Blow Lower	*Length (mm)*	15.08	18.03	16.468	0.97	5.92
	Weight (mg)	45.3	66.5	55.9	6.70	11.99
Blow Higher	*Length (mm)*	14.15	18.09	16.253	1.08	6.63
	Weight (mg)	48	79.6	63.6	9.70	15.25
Caffeine Lower	*Length (mm)*	14.42	18.23	16.6405	1.22	7.31
	Weight (mg)	46.8	77.8	67	9.30	13.88
Caffeine Higher	*Length (mm)*	14.93	18.42	16.925	1.10	6.53
	Weight (mg)	63.9	79.3	73.1	3.80	5.20
Control	*Length (mm)*	13.84	18.09	15.761	1.35	8.55
	Weight (mg)	69.4	98.6	88.1	6.60	7.49
Paracetamol Lower	*Length (mm)*	11.91	14.75	13.372	0.84	6.31
	Weight (mg)	35.5	43.1	39.9	2.20	5.51
Paracetamol Higher	*Length (mm)*	11.16	13.46	12.44	0.77	6.20
	Weight (mg)	36.9	43.9	40.9	2.10	5.13

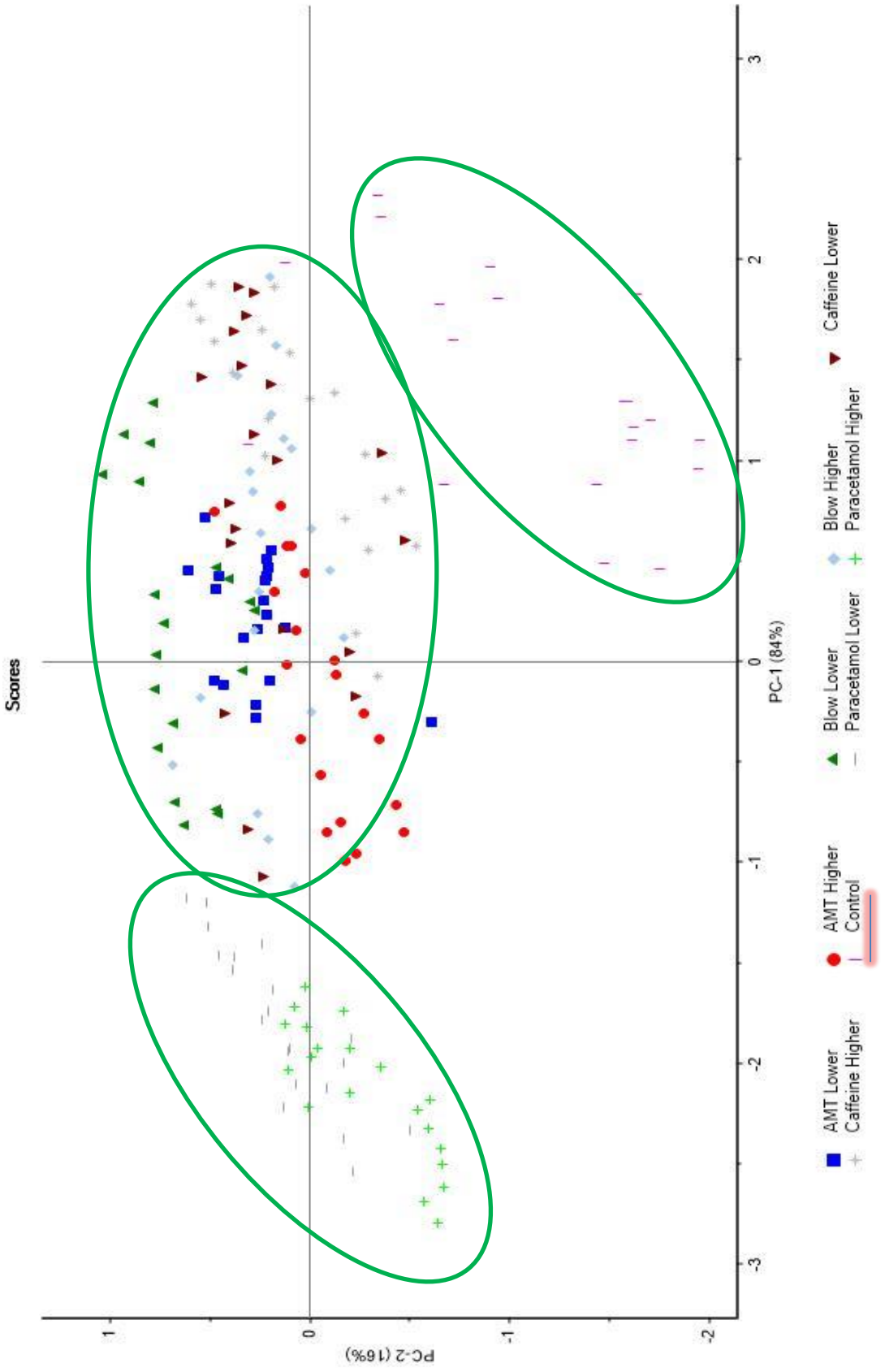


Figure 129: PCA plot showing selected drugs and dosages on day 4. The reader is referred to Table 8 for concentrations.

Day 4- Discussion

An overview of day 4 predominantly shows dosage differences when observing larval length as opposed to weight. Sometimes results are not shown as significantly different but the range of results will show a trend. Higher doses of AMT and Blow still appear to be inhibiting development but the result is no longer significant. Higher caffeine doses appear to encourage larval development further and higher paracetamol doses inhibit development further.

Day 6

Larval Length

AMT dosages are still both shown to be significantly above the control population at sampling day 6 ($P<0.001$) and a difference is also shown between the two doses ($P<0.01$), (Figure 130). The higher dose has also allowed acceleration of development but impeded it slightly when comparing with the lower dose. Blow is also shown as significantly larger than the control ($P<0.001$) but at this point dosage does not differ this result. Caffeine follows the same trend seen at day 4 where the larger measurements are attained with a higher dosage; however, the lower dosage is also shown to cause a significant length difference. Paracetamol had not previously shown any difference against the control when analysed together. The doses separately appear to result in the same developmental changes, the lower dose results in a few higher length measurements but the mean length appears similar, (Table 22).

Larval Weight

AMT dosage at this time point had no effect on larval weight; results were still significantly larger than the control. No significant difference was observed between the two dosages in relation to larval weight. Blow showed the higher dose resulted in measurements closer to the control while the lower dose had accelerated growth ($P<0.001$). The difference seen between the dosages of caffeine is large as shown in Figure 131. The difference between both the two doses and the caffeine samples against the control were significantly different ($P<0.001$) and the higher dose encouraged acceleration of development quite significantly. Paracetamol originally showed a significant increase in weight when analysing the doses together. Separate analysis showed a very similar range of data but with very different spreads of measurements. It was shown that the higher dosage resulted in measurements not too dissimilar to the control, whereas the lower dosage, which should impede development less, remained significantly increased.

Principle component analysis plotting PC 1 vs PC 2 showed 100% of the data variance and continued to show clustering of the data points dependant on both the drug and dosage (Figure 132).

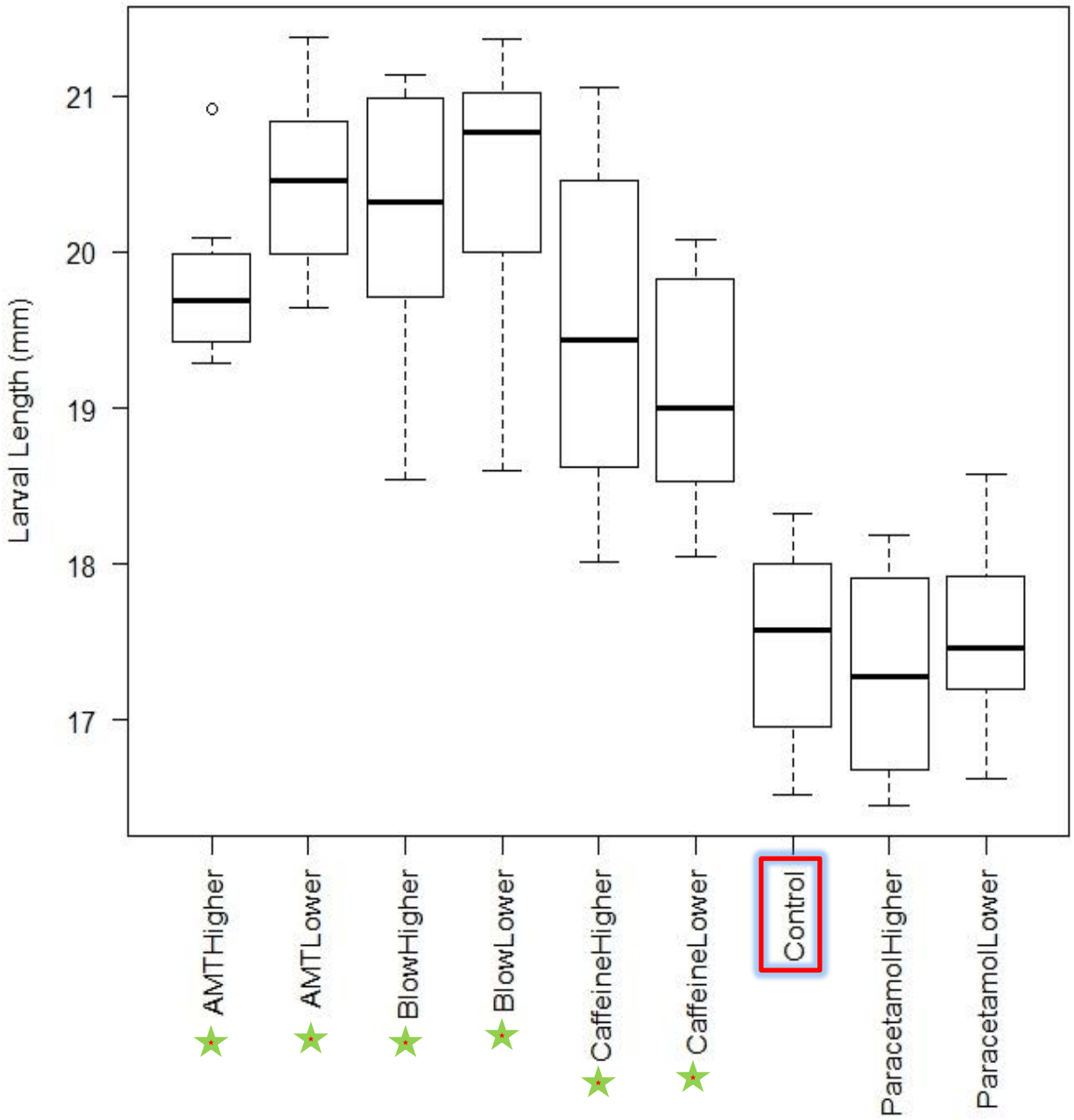


Figure 130: Box plot showing larval length for day 6 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

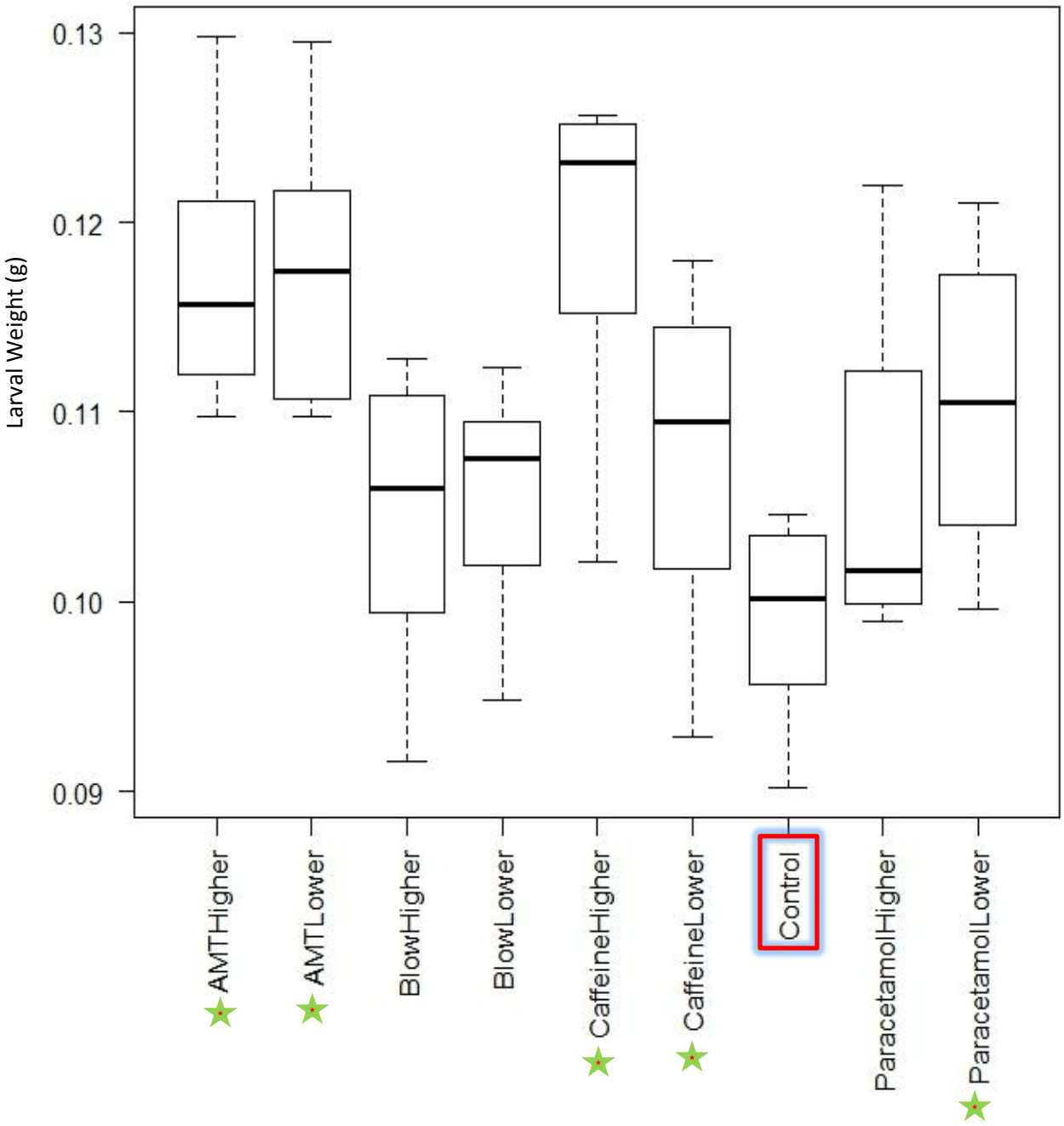


Figure 131: Box plot showing larval weight for day 6 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

Table 22: The minimum, maximum and mean lengths and weights (including standard deviation) on day 6 of selected drugs and dosages. The reader is referred to Table 8 for concentrations.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
AMT Lower	*Length (mm)*	19.64	21.38	20.435	0.56	2.76
	Weight (mg)	109.8	129.5	117.1	6.20	5.29
AMT Higher	*Length (mm)*	19.29	20.92	19.7335	0.38	1.93
	Weight (mg)	109.8	129.8	116.9	5.90	5.05
Blow Lower	*Length (mm)*	18.6	21.37	20.4285	0.88	4.32
	Weight (mg)	94.8	112.3	106.1	4.70	4.43
Blow Higher	*Length (mm)*	18.54	21.14	20.221	0.85	4.21
	Weight (mg)	91.6	112.8	104.5	7.40	7.08
Caffeine Lower	*Length (mm)*	18.05	20.08	19.073	0.70	3.65
	Weight (mg)	92.9	118	108.4	7.30	6.73
Caffeine Higher	*Length (mm)*	18.01	21.06	19.4965	1.05	5.40
	Weight (mg)	102.1	125.6	119.4	7.50	6.28
Control	*Length (mm)*	16.52	18.32	17.484	0.58	3.31
	Weight (mg)	90.2	104.6	98.9	4.80	4.85
Paracetamol Lower	*Length (mm)*	16.62	18.57	17.5505	0.54	3.09
	Weight (mg)	99.6	121	110.6	7.20	6.51
Paracetamol Higher	*Length (mm)*	16.45	18.18	17.3015	0.63	3.63
	Weight (mg)	99	121.9	105.4	6.90	6.55

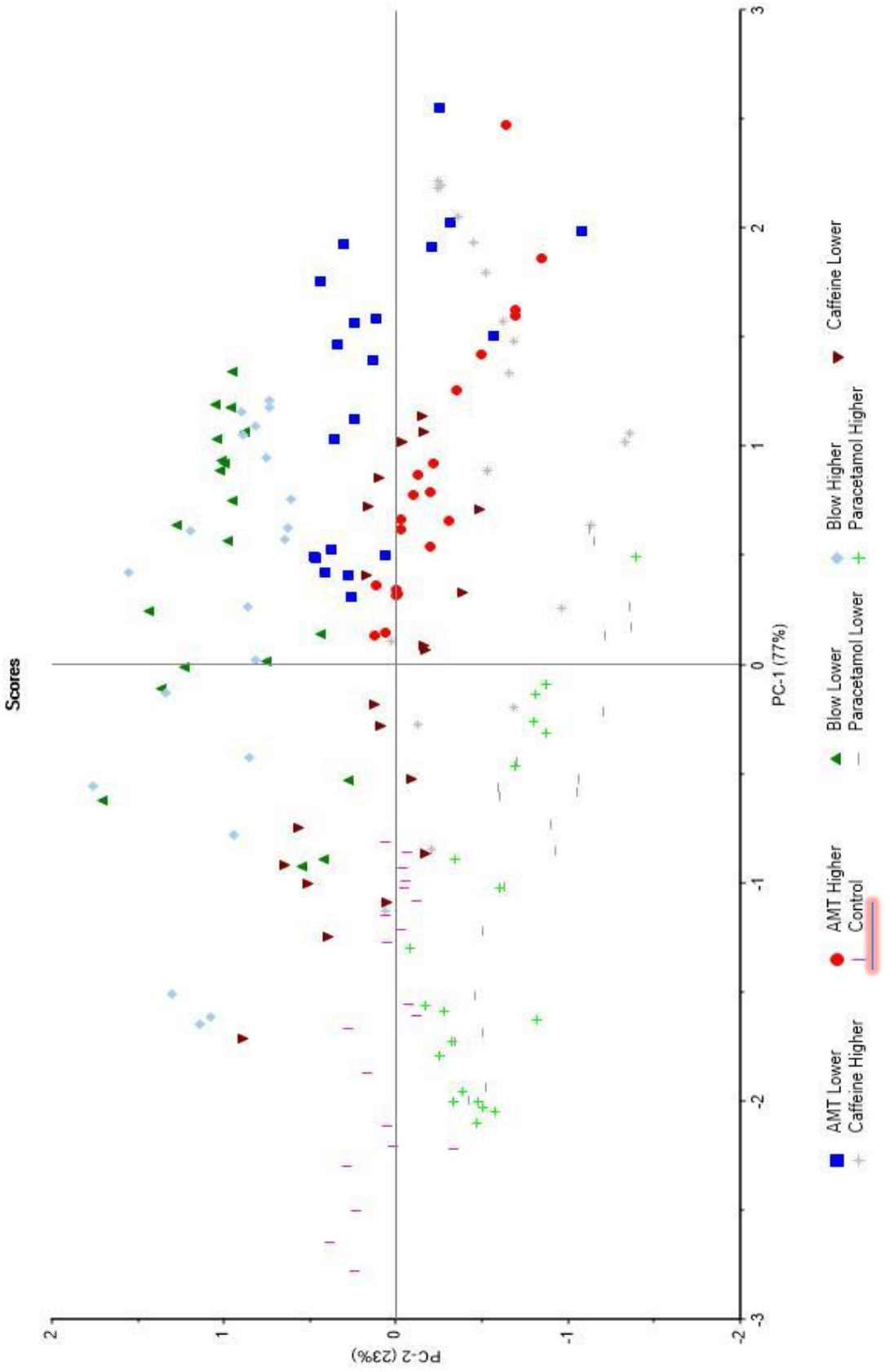


Figure 132: PCA plot showing selected drugs and dosages on day 6. The reader is referred to Table 8 for concentrations.

Day 6- Discussion

It is clear that dosage can considerably alter the results of larval developmental studies. AMT and Blow, on the whole accelerate growth. The higher dosages appear to allow a certain amount of acceleration (the drug effect on development) while also impeding (the drug effect on palatability). It could be that the drug itself speeds up development or behaviour but the larvae are feeding less when a higher concentration of the drug is present. Caffeine and Paracetamol provide clearer results. Development as a whole is increased by caffeine, the higher dose within this study produces higher masses and larger lengths. Paracetamol development is impeded, however the larger concentration impeded more, resulting in the more statistically different responses that are observed.

Day 8

Larval Length

Results from the population given AMT are all shown to not be significantly different, even the different dosages are shown to statistically similar. Analysis of the boxplot shows that the lower dosage results in a much larger spread of data but with a similar mean (Figure 133). Blow shows the same trend, no results are significantly different, including between the two dosages. This is suspected to be due to larval shrinkage and this happens prior to pupariation. A larger range of data measurements is shown for the higher dosage of Blow. Further investigation is required into the effect of different drug concentrations on larval uptake. It is possible that if larval ingestion were delayed due to

the diet being unpalatable, then the effect of the drug, whether that is acceleration or delay, would be decreased as the uptake is decreased.

Caffeine has not only accelerated in terms of physical measurements but also development stage, these larvae are now reducing in size to prepare for pupariation. The larvae feeding on a higher dosage are significantly different ($P<0.01$) to the lower dosage, as shown before. There are no significant results shown between the control and Paracetamol populations or between the two Paracetamol dosages. The two sets of dosage data are however skewed differently. The higher dose has lower values and a lower mean length as larvae are more impeded in their development, the lower dose has more high measurements and a higher mean length as it is less impeded in its growth and development.

Larval Weight

AMT weight shows the largest difference to the control when at a lower dosage at this time point, the higher dosage remains insignificant. Blow also shares this trend with the largest differences to the control showing at a lower dosage ($P<0.001$). Caffeine is significantly different at its higher dose but similar to the control at its lower dose. The population with a caffeine diet appear to be preparing to pupate and therefore weight and length will decrease, however the higher dosage still appears accelerated. When analysing both dosages of the Paracetamol population together a significant increase in weight was shown. Individual analysis of the dosages show that both dosages are significant but the higher dose is less different to the control samples. Both have stunted development and are developing slower but the higher is still more impeded. They are also statistically different from each other ($P<0.01$).

Principle component analysis again shows clustering of individual drugs and dosages as both measurements are different enough for this variation to be depicted.

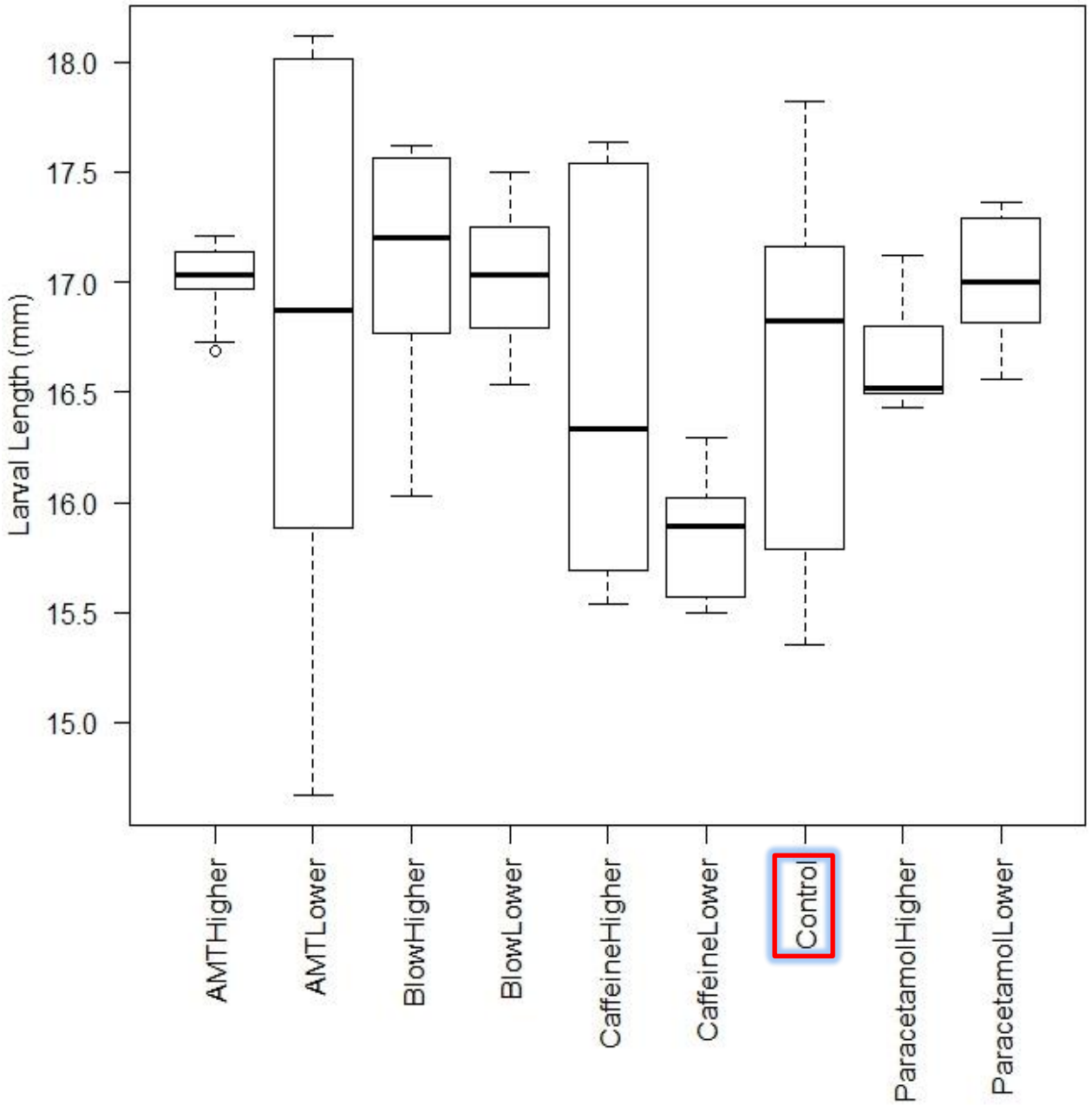


Figure 133: Box plot showing larval length for day 8 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

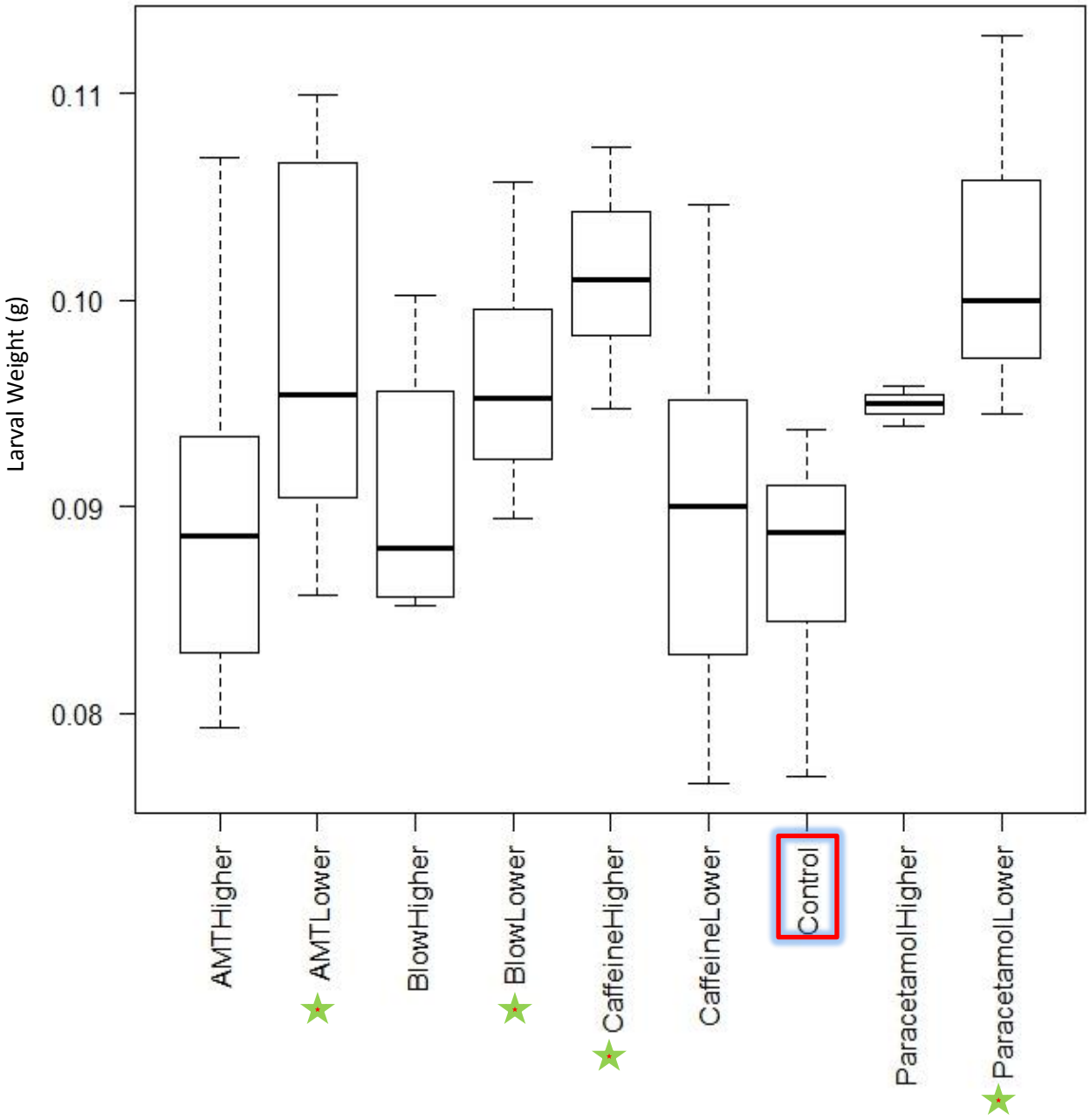


Figure 134: Box plot showing larval weight for day 8 across selected samples at different doses. The reader is referred to Table 8 for concentrations.

Table 23: The minimum, maximum and mean lengths and weights (including standard deviation) on day 8 of selected drugs and dosages. The reader is referred to Table 8 for concentrations.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
AMT Lower	*Length (mm)*	14.67	18.12	16.749	1.26	7.52
	Weight (mg)	85.7	109.9	97.7	8.30	8.50
AMT Higher	*Length (mm)*	16.69	17.21	17.017	0.15	0.89
	Weight (mg)	79.3	106.9	89.1	7.20	8.08
Blow Lower	*Length (mm)*	16.538	17.5	17.01	0.31	1.80
	Weight (mg)	89.4	105.7	96.5	5.40	5.60
Blow Higher	*Length (mm)*	16.031	17.62	17.135	0.44	2.58
	Weight (mg)	85.2	100.2	90.8	6.00	6.61
Caffeine Lower	*Length (mm)*	15.5	16.29	15.836	0.26	1.61
	Weight (mg)	76.6	104.6	89.3	8.20	9.18
Caffeine Higher	*Length (mm)*	15.539	17.64	16.497	0.88	5.36
	Weight (mg)	94.7	107.4	101	4.00	3.96
Control	*Length (mm)*	15.35	17.82	16.5415	0.80	4.86
	Weight (mg)	76.9	93.7	87.3	4.80	5.50
Paracetamol Lower	*Length (mm)*	16.556	17.364	17.002	0.27	1.60
	Weight (mg)	94.5	112.8	101.4	5.50	5.42
Paracetamol Higher	*Length (mm)*	16.43	17.12	16.654	0.24	1.45
	Weight (mg)	93.9	95.8	94.9	0.60	0.63

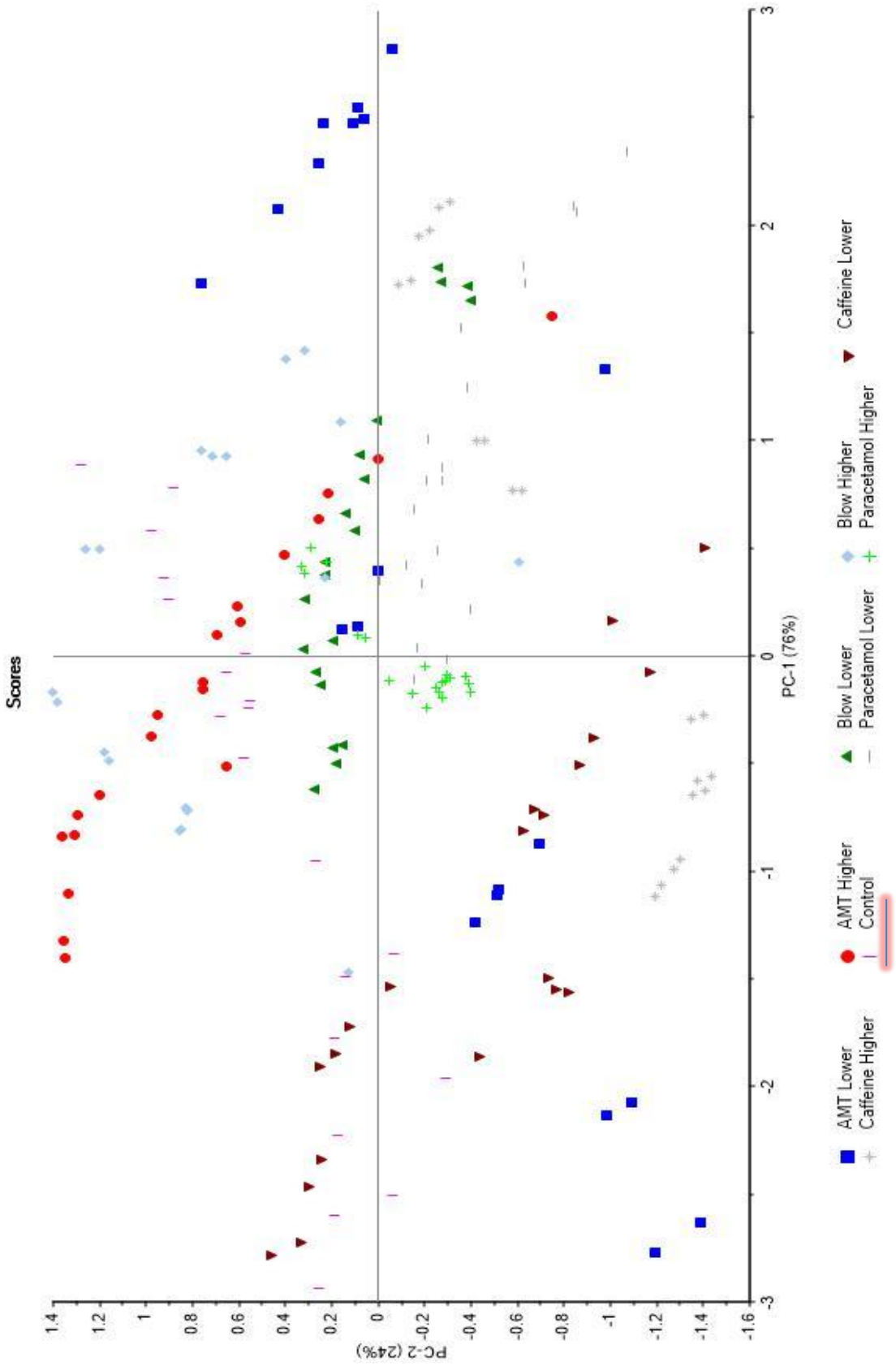


Figure 135: PCA plot showing selected drugs and dosages on day 8. The reader is referred to Table 8 for concentrations.

Day 8- Discussion

All diets continue with the previously observed trends, Caffeine and Paracetamol behave as hypothesized. Higher doses of compounds known to accelerate development will enable the larvae to accelerate even more. Higher doses of compounds known to delay development will enable further delays for the blowfly development.

AMT and Blow both appear to show accelerated development because of the lower concentration of the two tested. This may be due to a lower level of ingestion, possibly because of decreased palatability. Further investigation is required to confirm levels where palatability may be compromised and enable determination of this trend.

It can be concluded that dosage of a compound, in those discussed in this section, do invariably affect developmental rate and because of this, are of interest in relation to estimation of minimum post mortem interval.

5.2.4.1 Overall Dosage discussion

Whilst the importance of acknowledging drug presence has been demonstrated previously in this chapter, the influence of concentration however, is also significant. Concentration can vary in multiple scenarios associated with a crime scene. The dose taken prior to death is one of the major variables; it may be in larger doses having potentially contributed to the cause of death, or lower doses. Once a drug enters the body, metabolism and elimination is initiated. Presence and availability of the parent drug and its metabolites will therefore depend upon progression of these processes. Research has also shown that the level of decomposition can also modify drug concentration [308], within porcine tissues the concentration of drug analysed increases, with an increase in the state of decomposition. The observed increase differed dependant on the drug. The concentration present may also be dependent on the tissue of choice, one author found concentrations to be similar within the lungs, muscle, kidney, liver and heart, higher within the spleen and vitreous and lower in the fat tissue of animals [147]. Another author communicates that generally concentrations found within the liver are higher than those found in the muscle [150]. It must also be noted that concentrations investigated in publications often use the lethal dose of their animal model, which is not necessarily equivalent to the dosage encountered within humans.

If the concentration of a drug is shown to vary the developmental effects, then the concentration becomes equally as important to acknowledge during the estimation of PMI. Malathion was shown to not affect larval length differently when various doses were researched [125]. This was also concluded in a study using five different concentrations of Paracetamol [304], and a review on codeine doses [306]. Research using different

concentrations of cocaine however determined that larval growth was significantly more rapid in colonies provided with higher doses compared with lower doses and a control [153]. Another study showed three different morphine concentrations and stated that although the control and lower dosage showed similar developmental rates while the higher doses delayed development [148].

Differences were shown between all drugs in this study, researched at their different dosages. Four drugs were discussed within this chapter with some very significant differences. AMT and Blow both appear to show that development rate is increased when compared to the control. Separate analysis actually shows the lower of the two concentrations accelerates growth most while the higher concentration accelerates growth in comparison with the control but not as significantly as the lower, this could potentially be due to delays in ingestion of the diet because of palatability changes owed to the drug presence.

Caffeine again showed that development rate was accelerated and then in relation to dosage presented the highest dose to stimulate growth further than the lower. Paracetamol delayed development, separate analysis of the dosages showed the lower dose to delay development less than the higher dose, and in fact, the lower dosage actually pupariated earlier than the control population.

In conclusion, it appears that the developmental rate is also dependant on the drug dosage as well as its presence and must be considered during PMI estimations.

5.2.5 Does the observed effect differ between species?

It is hypothesized that species show the effects of drugs on development differently [148],[284]. An experiment was therefore carried out to determine if, for two drugs of interest, a similar development pattern would be shown, when comparing *Lucilia sericata* to *Calliphora vicina* development patterns. 6APB and AMT were selected at random for this study, from drugs previously shown to have a developmental effect and were run alongside a control diet; diets were prepared as previously explained.

5.2.5.1 Pupariation time and instar

As before, instar was recorded at 12-hour intervals, this was used to track progression of development. This data is shown in a bar chart in Figure 136 . Instars 1-3 were shown to have no difference when compared to the control. This changed for the post-feeding stage as the control spent an extra 24 hours in the post-feeding stage prior to pupariation when compared with the populations with drug additions, (Figure 136).

When comparing previous results from *C.vicina* (Figure 81 and Figure 82) to the results for *L.sericata*, the same pattern can be seen, suggesting that, in relation to development time and pupariation *C.vicina* and *L.sericata* react in the same way to selected dosages of AMT and 6APB.

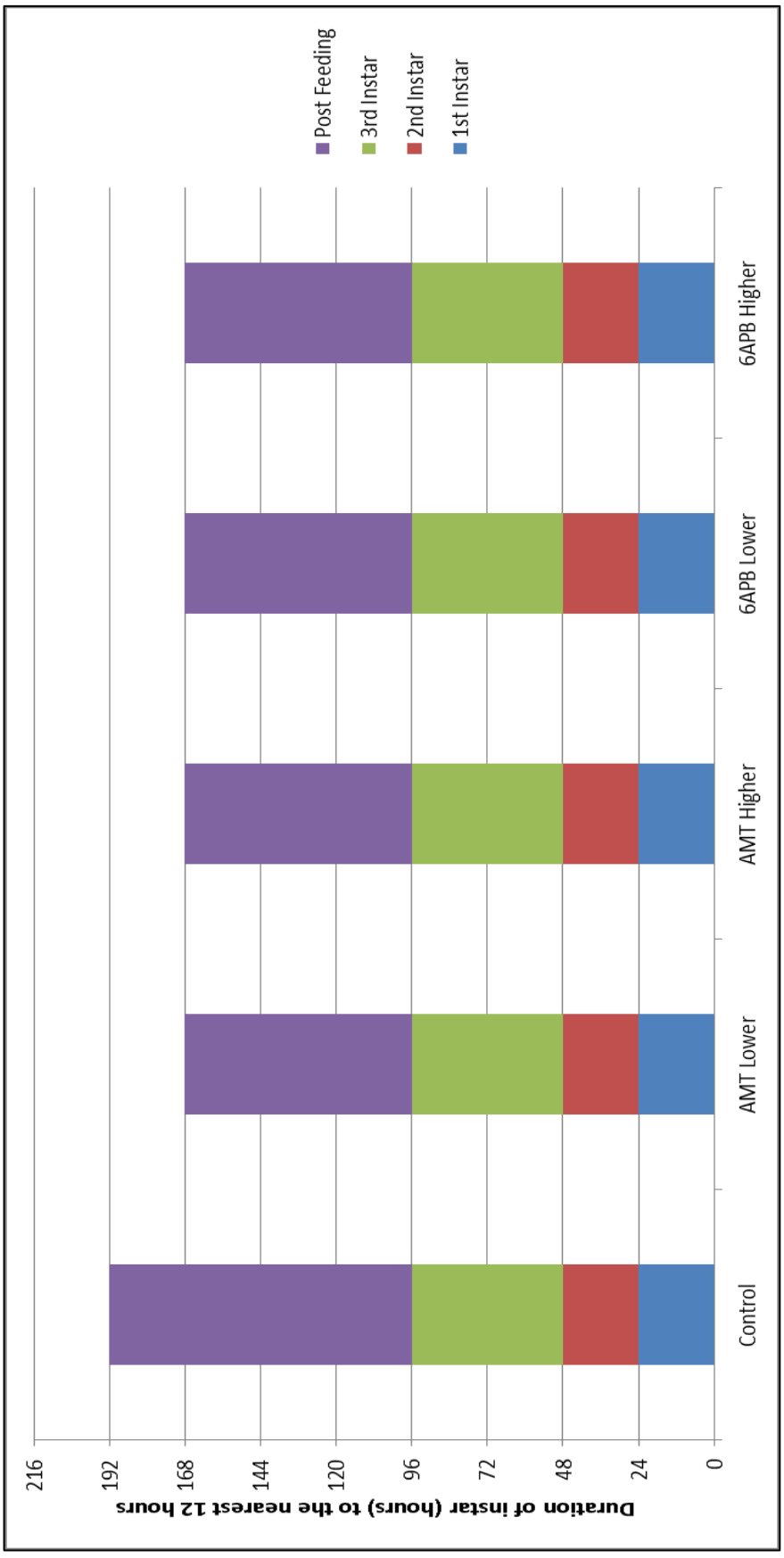


Figure 136: Bar chart showing the duration of each instar during the developmental time of blowfly larvae in the presence of NPS for *L.sericata*. The reader is referred to Table 8 for concentrations.

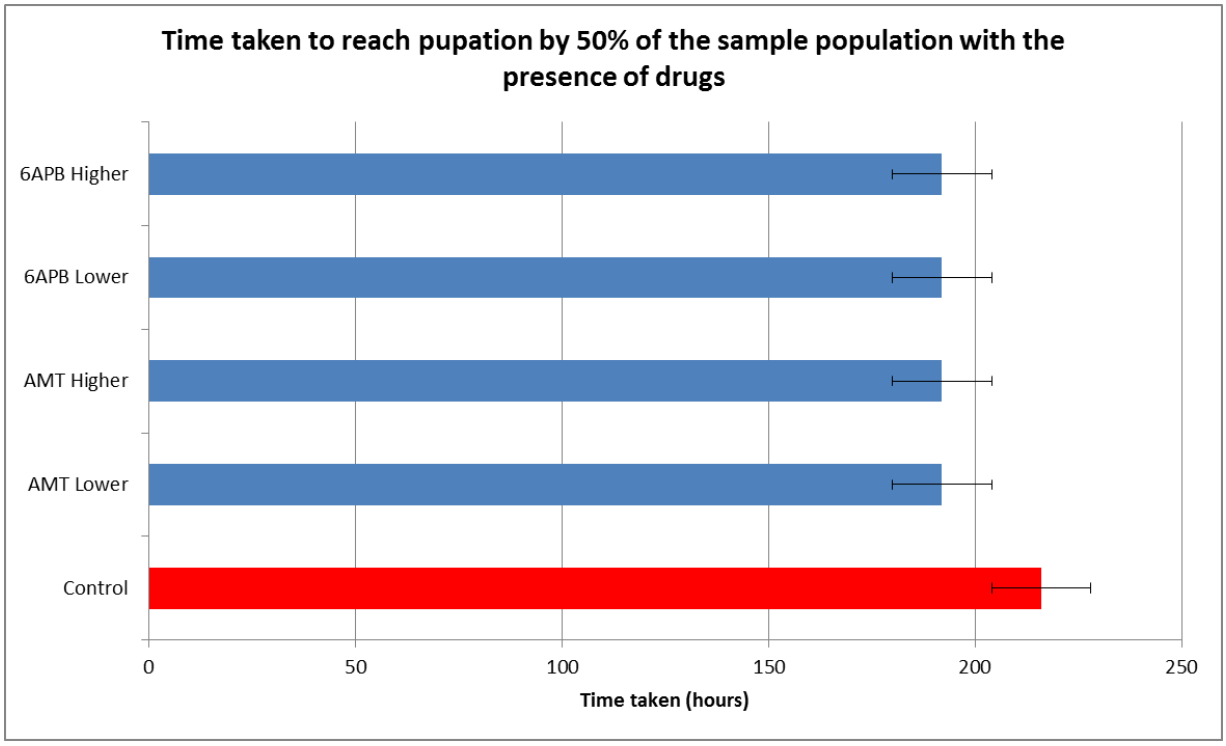


Figure 137: Bar chart showing time taken for 50% of the population to reach pupariation for each drug diet, including error bars for *L.sericata*. The reader is referred to Table 8 for concentrations.

An overview of the development of both larval length and weight for *L.sericata* is shown in Figure 138 and Figure 139. These appear to change dependant on the drug presence. AMT is shown to reach the largest larval length and weight when comparing to 6APB and control. The developmental rate for all three diets is shown to increase up to day 5, after which data plateaus or decreases. Differences are shown in comparison with the control. Developmental trends shown in the figures are extremely similar to those observed in *C. vicina.*

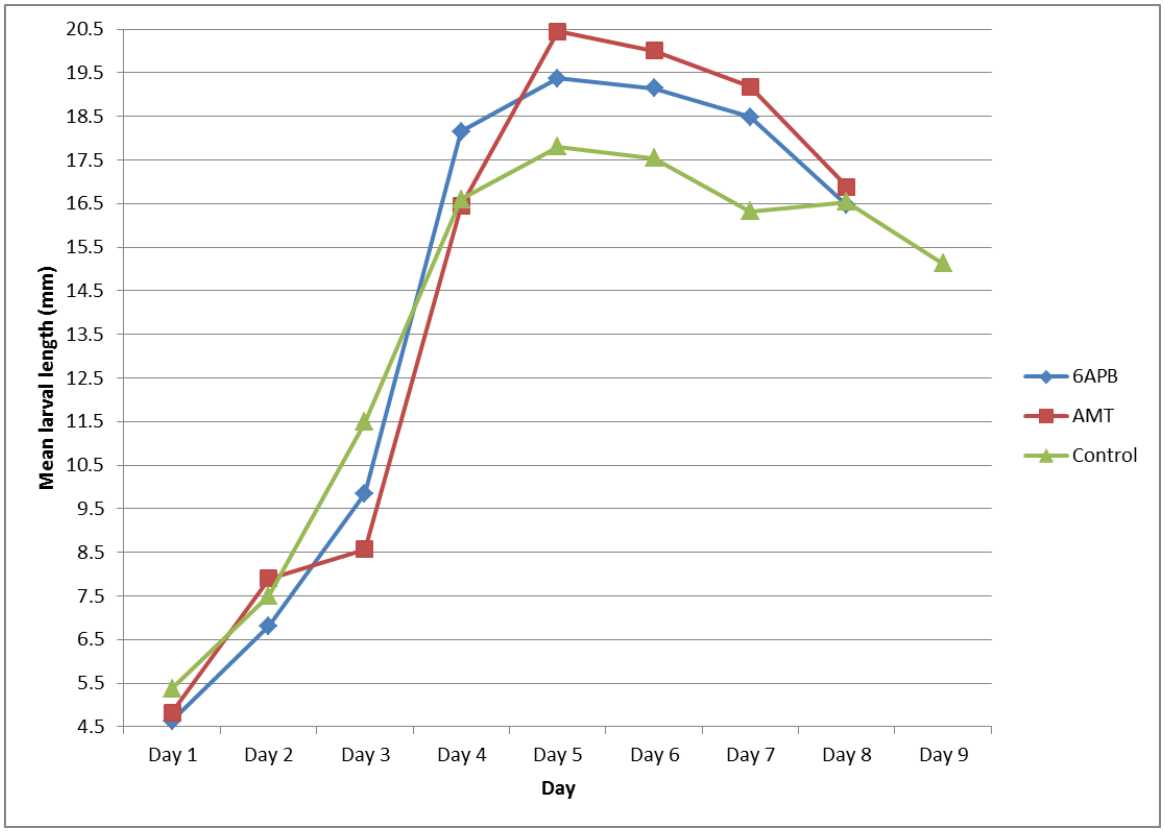


Figure 138: Line graph showing an overview of mean larval length for *L.sericata* across 9 days.

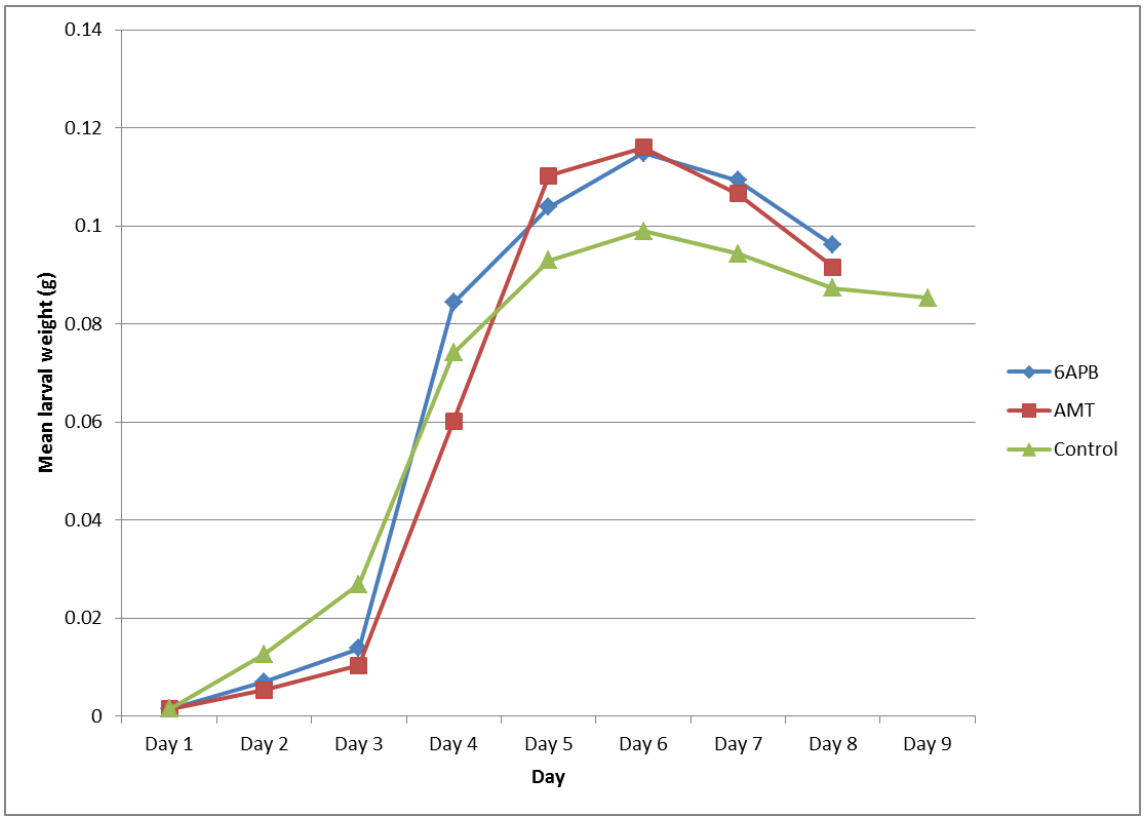


Figure 139: Line graph showing an overview of mean larval weight for *L.sericata* across 9 days.

L. sericata- Day 1

Larval lengths on day 1 (Figure 140) ranged between 3.51 mm and 7.05 mm. A mixed effects model showed significant differences and a Tukey test showed that 6APB was smaller (P=0.0105) than the control, as was AMT (P=0.0739).

Larval weights on day 1 (Figure 141) ranged between 0.0007 g and 0.003 g. No differences were shown between these groups for day 1. Development data is shown in Table 24.

Principle component analysis (Figure 158) showing 100% of available data variance did not show data to cluster well. This was to be expected due to the small amount of time since larvae had been placed on the diet.

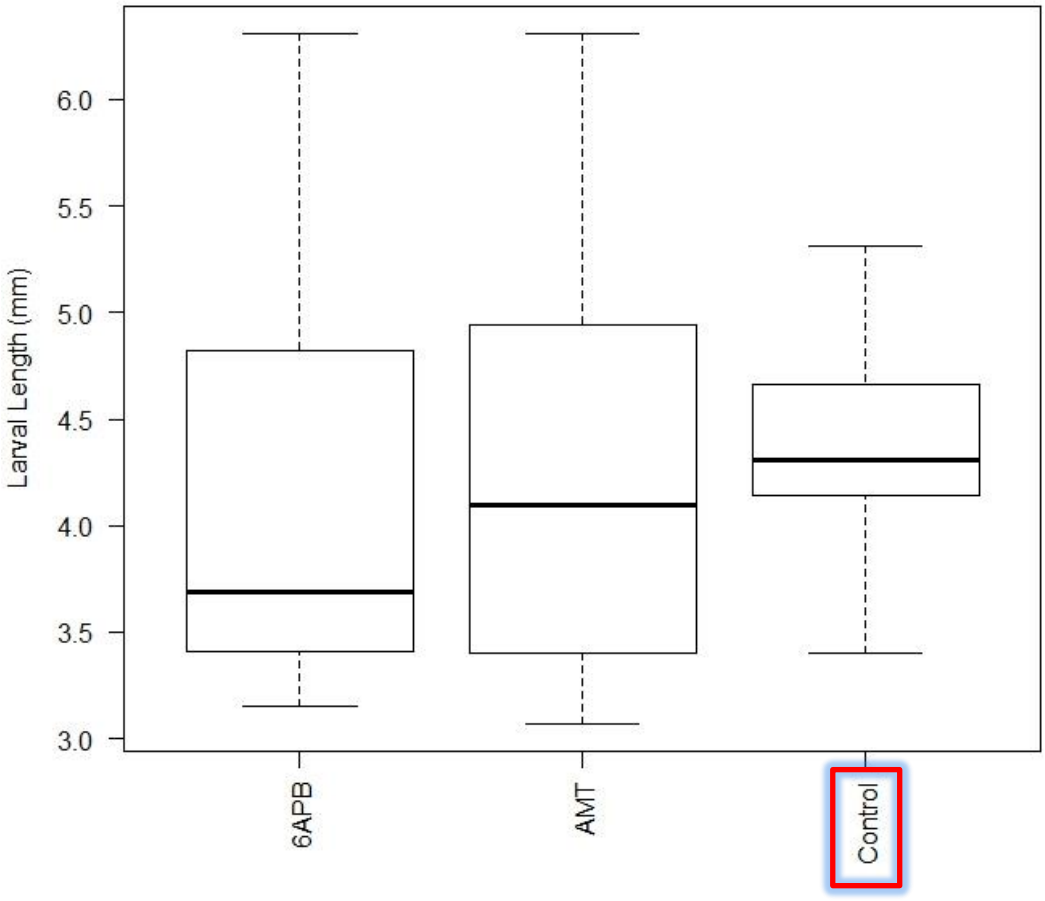


Figure 140: Box plot showing larval lengths for day 1 *L.sericata* across selected samples.

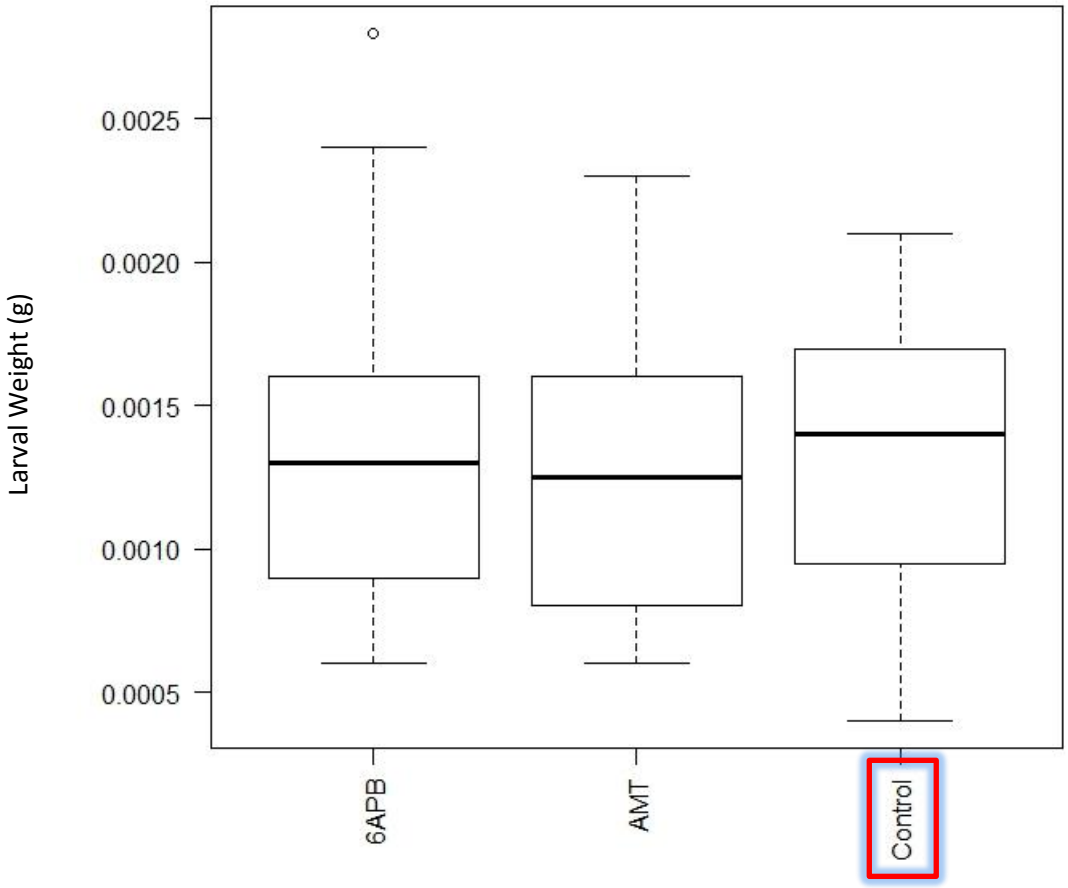


Figure 141: Box plot showing larval weights for day 1 *L.sericata* across selected samples.

Table 24: The minimum, maximum and mean lengths and weights (including standard deviation) on day 1 of selected drugs for *L.sericata*.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	3.62	7.04	4.6403	0.93	20.01
	Weight (mg)	0.7	3	1.5	0.50	33.33
AMT	Length (mm)	3.51	7.04	4.823	0.95	19.65
	Weight (mg)	0.7	2.7	1.5	0.50	33.33
Control	Length (mm)	3.88	7.05	5.3825	1.01	18.84
	Weight (mg)	0.7	2.3	1.5	0.50	33.33

L. sericata, Day 1- Discussion

Larval length and weight data showed few differences at this stage. This is very similar to the observations formed on day 1 during analysis of *C.vicina.*

L. sericata- Day 2

Day 2 larval lengths ranged between 4.76 mm and 10.82 mm. No significant differences were shown between these diets at this stage (Figure 142).

Larval weights showed more differences at this sampling point. Data ranged between 0.0022 g and 0.0149 g. Both drug diets were shown to be significantly smaller than the control (P<0.001) (Figure 143). Development data is shown in Table 25.

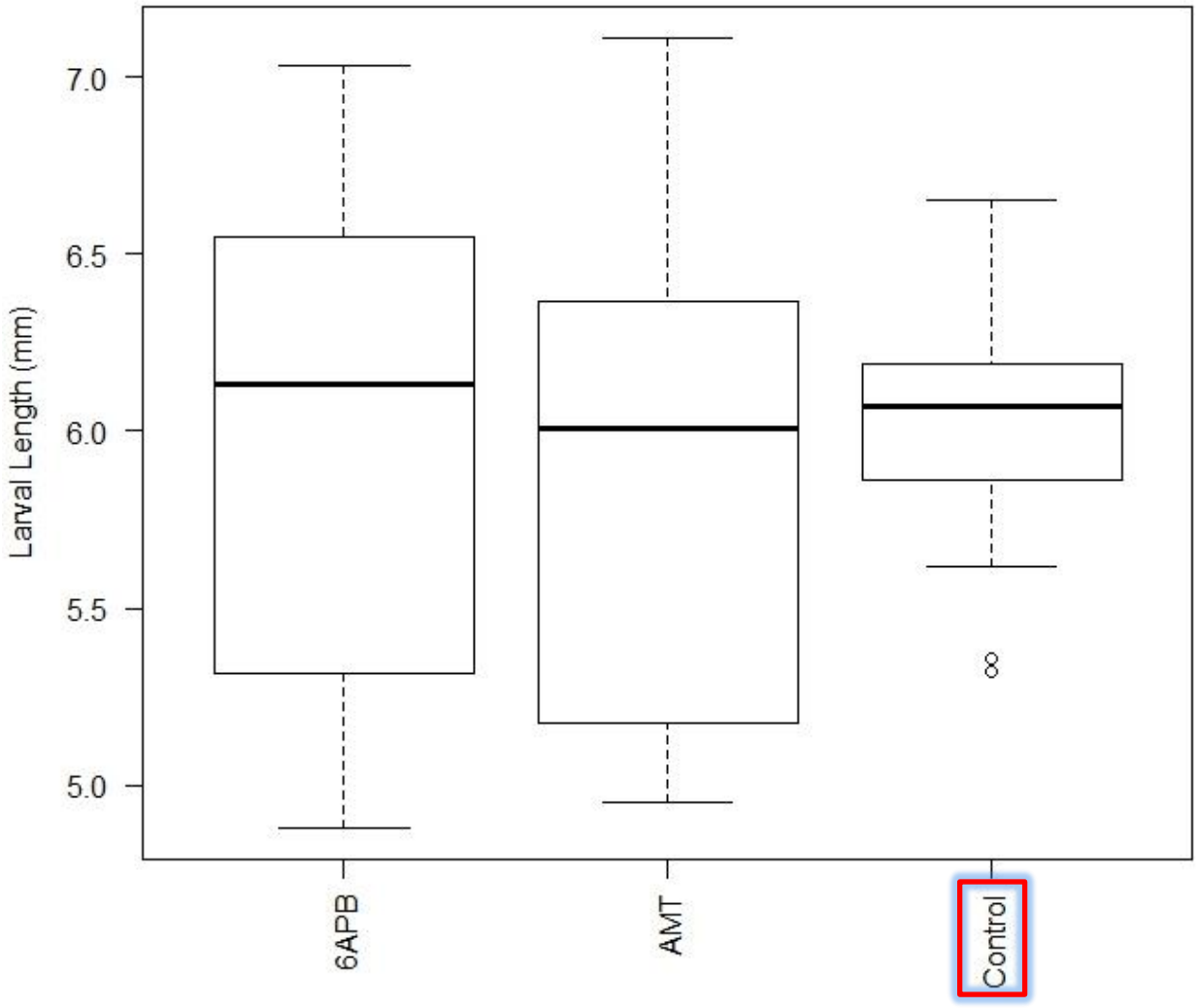


Figure 142: Box plot showing larval lengths for day 2 *L.sericata* across selected samples.

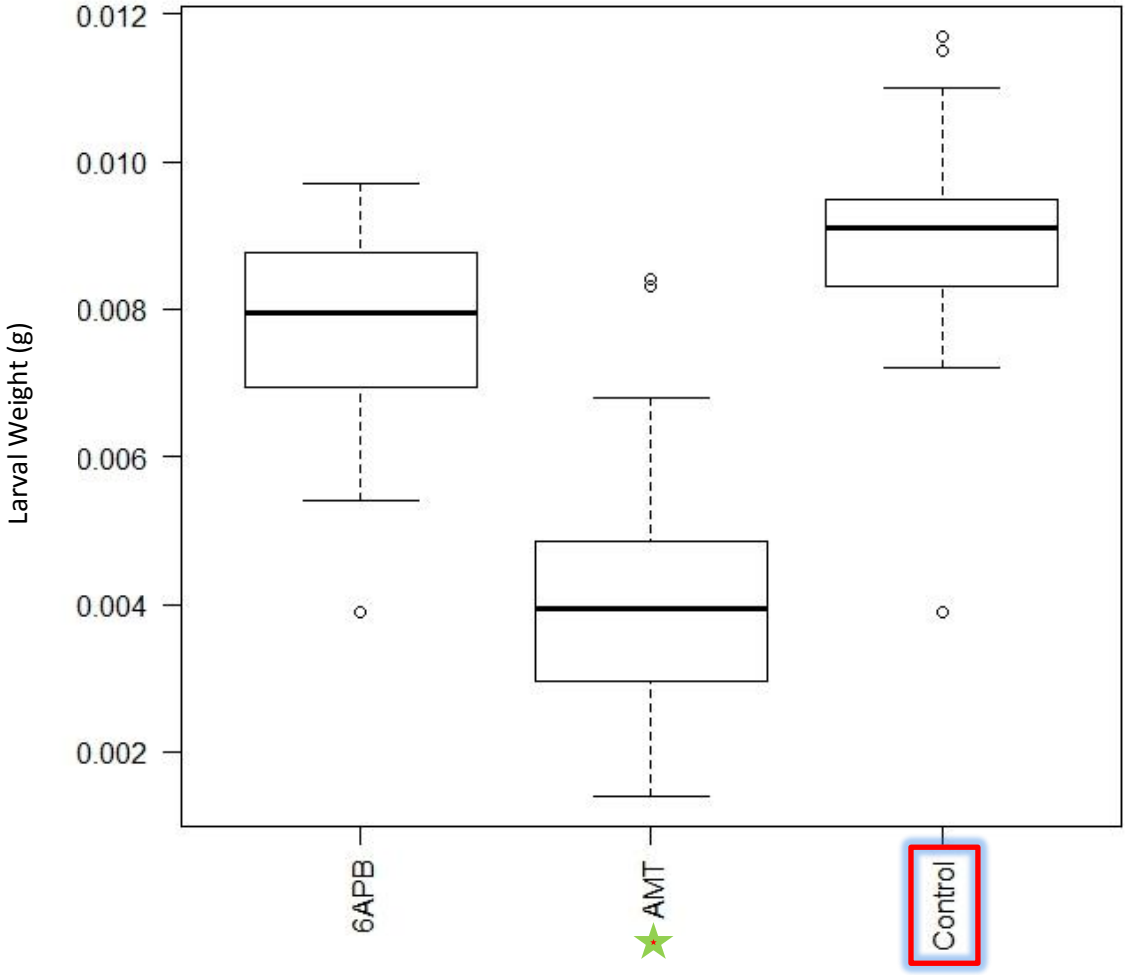


Figure 143: Box plot showing larval weights for day 2 *L.sericata* across selected samples.

Table 25: The minimum, maximum and mean lengths and weights (including standard deviation) on day 2 of selected drugs for *L.sericata.*

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	5.62	7.91	6.8125	0.67	9.82
	Weight (mg)	3.5	10.3	7	2.00	28.57
AMT	Length (mm)	4.76	10.82	7.9005	1.89	23.90
	Weight (mg)	2.2	10.9	5.4	2.20	40.74
Control	Length (mm)	6.66	8.24	7.501	0.55	7.31
	Weight (mg)	10.8	14.9	12.7	1.40	11.02

L. sericata, Day 2- Discussion

The effects discussed were also seen in analysis of *C.vicina*, where a decrease in larval weight was shown at 48 hours but larval length was indifferent, most likely due to drug presence.

L. sericata- Day 3

Larval length at day 3 ranged between 6.67 mm and 12.4 mm. Significant differences were shown between AMT and the control with larval lengths for AMT showing as significantly decreased (Figure 144). Larval weights ranged from 0.0042 g to 0.0315 g (Figure 145). Both drug diets were shown to be significantly smaller (P<0.001) than the control, perhaps symbolic of larvae avoiding drugged diets. Development data is shown in Table 26.

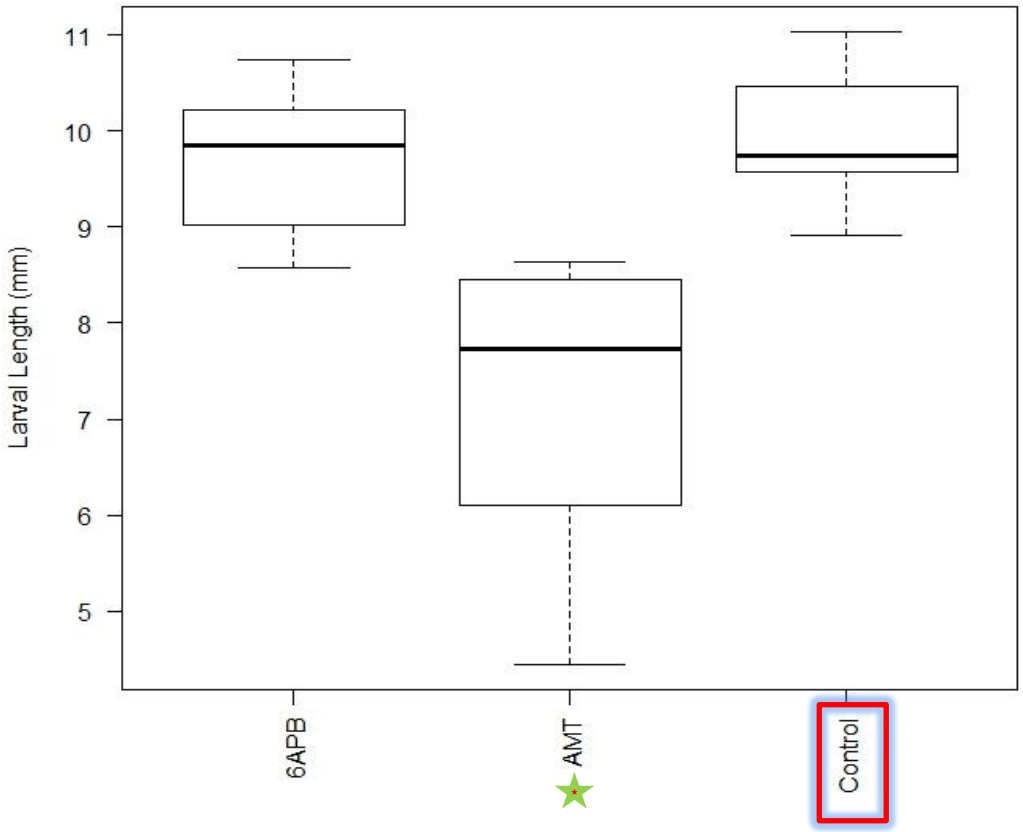


Figure 144: Box plot showing larval lengths for day 3 *L.sericata* across selected samples.

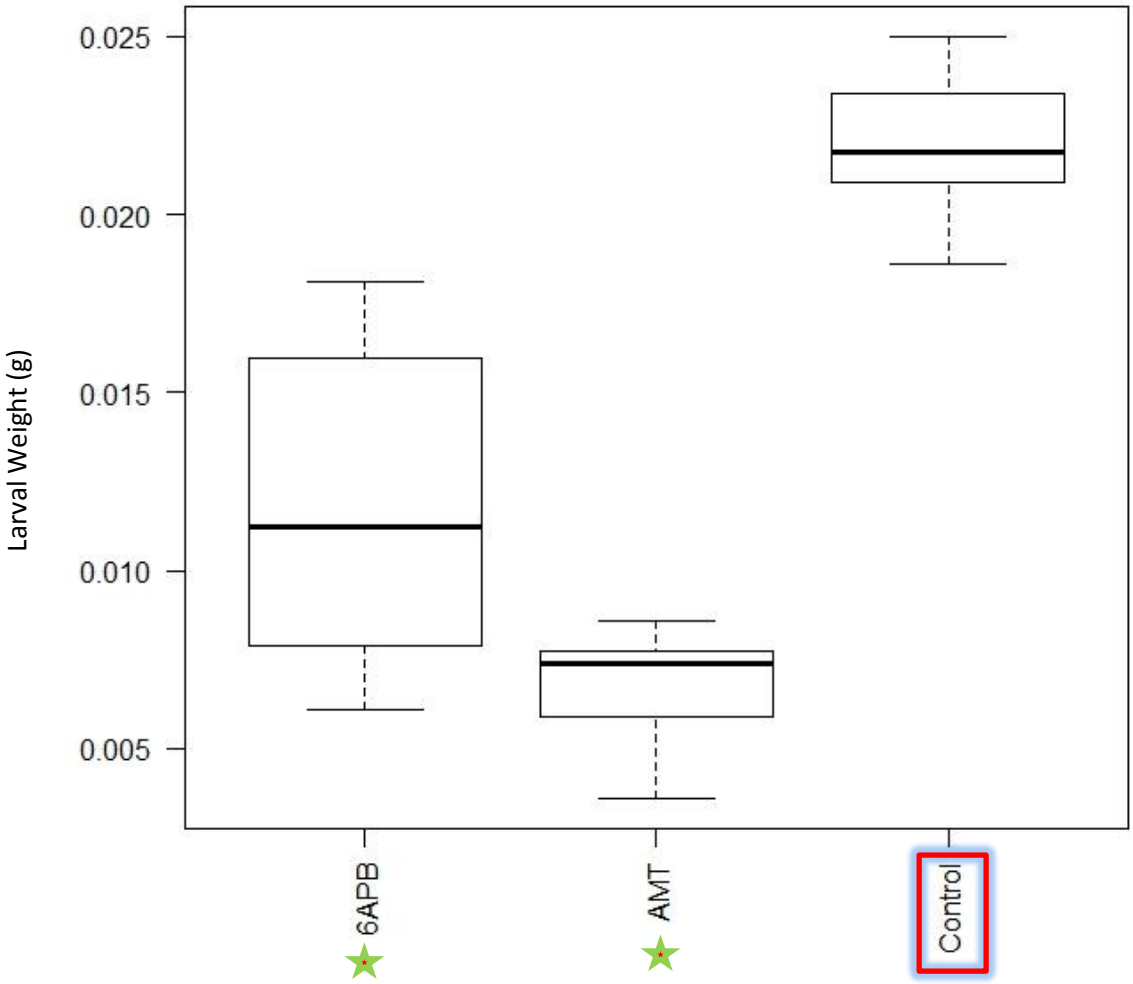


Figure 145: Box plot showing larval weights for day 3 *L.sericata* across selected samples.

Table 26: The minimum, maximum and mean lengths and weights (including standard deviation) on day 3 of selected drugs for *L.sericata*.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	8.66	10.88	9.8468	0.6517	6.62
	Weight (mg)	7.1	20.2	13.9	4	28.78
AMT	Length (mm)	6.67	12.4	9.77	1.7432	17.84
	Weight (mg)	4.2	16.3	10.4	3.1	29.81
Control	Length (mm)	10.67	12.17	11.4885	0.4978	4.33
	Weight (mg)	23.3	31.5	26.9	2.7	10.04

L. sericata, Day 3- Discussion

When compared with the data from the *C.vicina* species, the same trend is seen. This suggests that at this early stage, the drugs are affecting the species in the same manner.

L. sericata- Day 4

Larval length ranged between 14.03mm and 19.09mm at this sampling point. 6APB was shown to be significantly larger (P<0.001) than the control, whereas AMT was shown to be similar to the control (Figure 146). Weights ranged between 0.0393g and 0.106g. 6APB and the control population weighed very similar amounts whereas AMT weighed significantly less (P<0.001) (Figure 147). Development data is shown in Table 27.

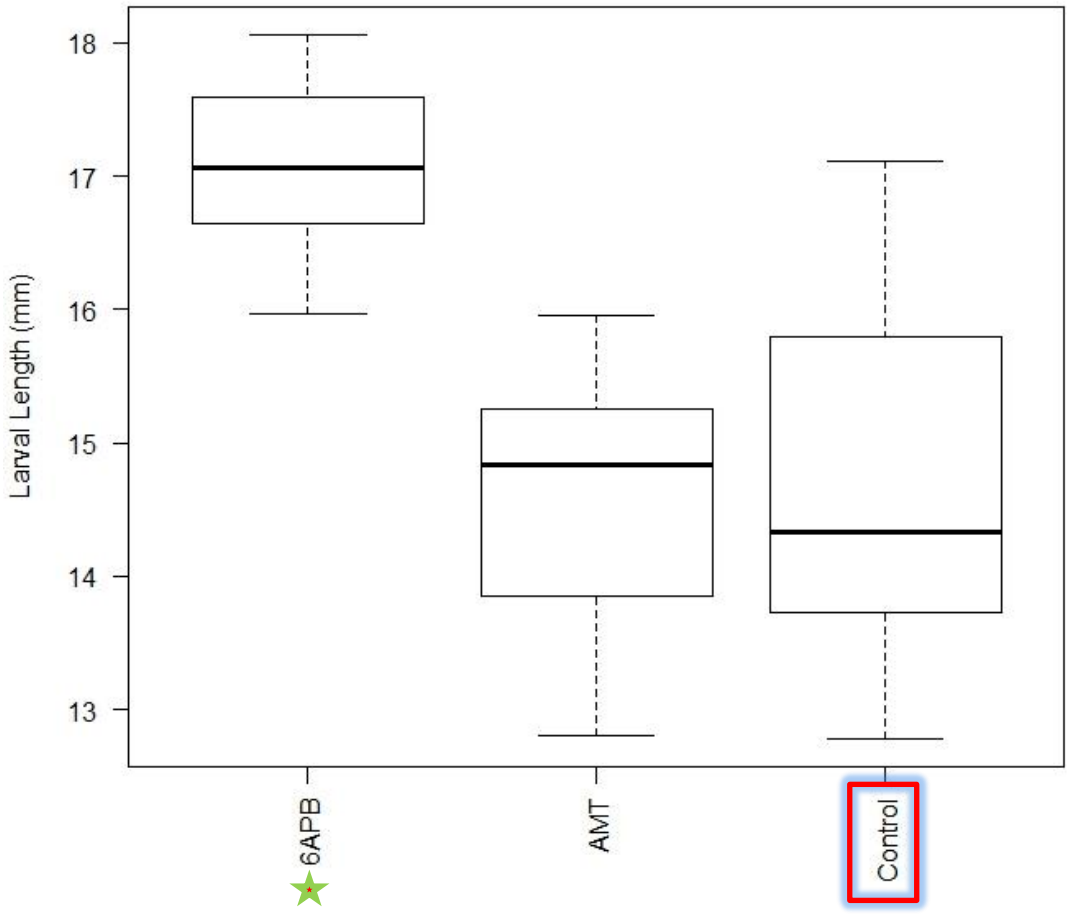


Figure 146: Box plot showing larval lengths for day 4 *L.sericata* across selected samples.

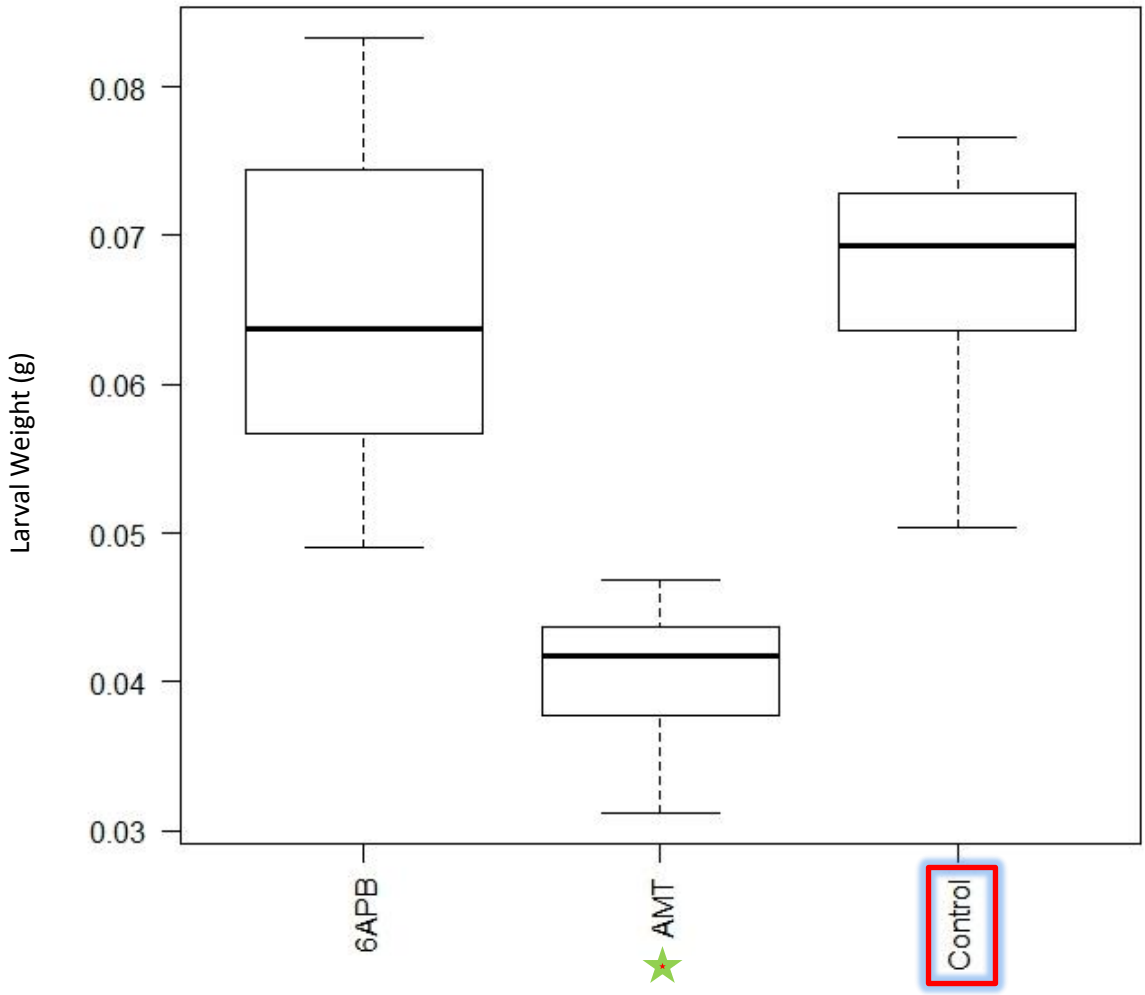


Figure 147: Box plot showing larval weights for day 4 *L.sericata* across selected samples.

Table 27: The minimum, maximum and mean lengths and weights (including standard deviation) on day 4 of selected drugs for *L.sericata*.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	16.99	19.09	18.1548	0.596	3.28
	Weight (mg)	61.8	106	84.4	11.7	13.86
AMT	Length (mm)	14.37	17.52	16.445	0.8639	5.25
	Weight (mg)	46.1	73	60.2	7.6	12.62
Control	Length (mm)	14.03	18.15	16.598	1.2806	7.72
	Weight (mg)	39.3	99.5	74.1	17.5	23.62

L. sericata, Day 4- Discussion

Trends at day 4 were again mirrored in the *C.vicina* data, AMT was however shown to be significantly larger at day 4 when compared in *C.vicina*, whereas it is not seen as a significant difference in this dataset.

L. sericata- Day 5

Larval lengths ranged between 16.33 mm and 18.8 mm on day 5 of sampling with both diets showing as significantly larger than the control population (P<0.001), this was also shown within the weight data as both drug diets were shown to be significantly larger and ranged between 0.0725 g and 0.1337 g (Figure 148,Figure 149,Table 28).

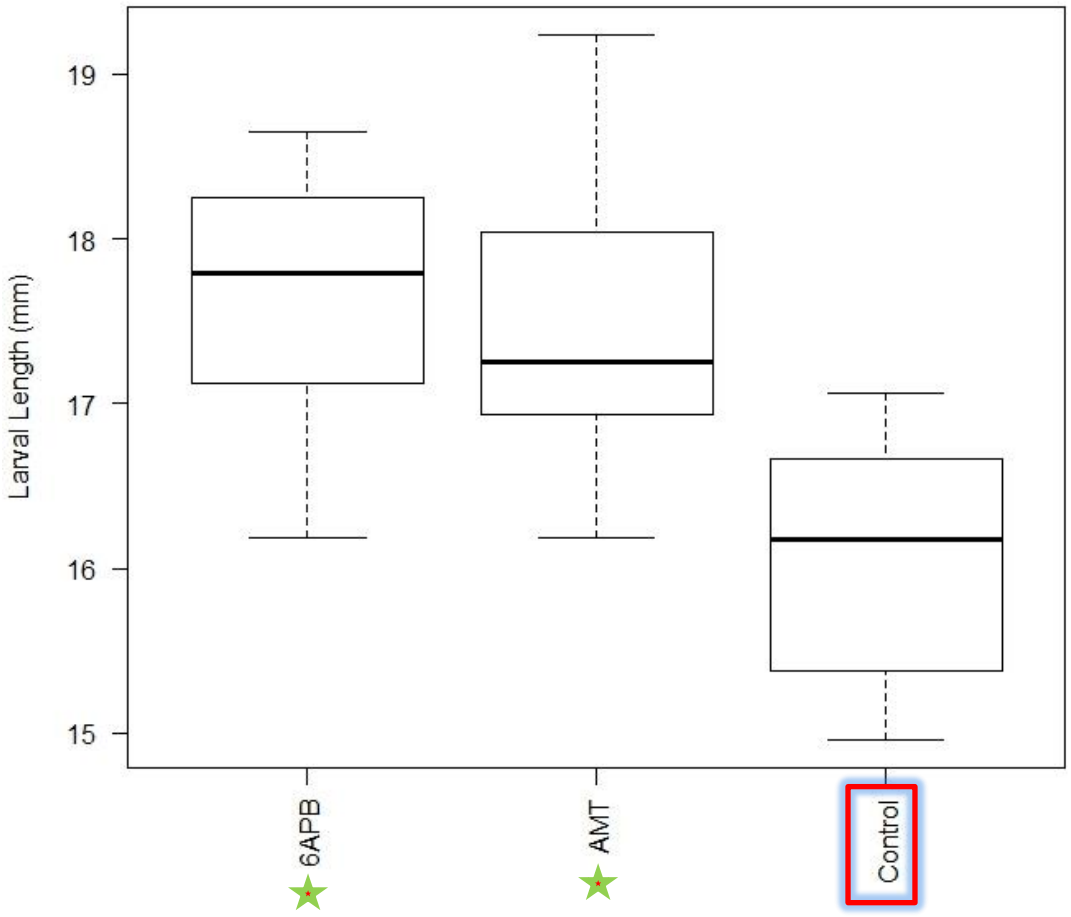


Figure 148: Box plot showing larval lengths for day 5 *L.sericata* across selected samples.

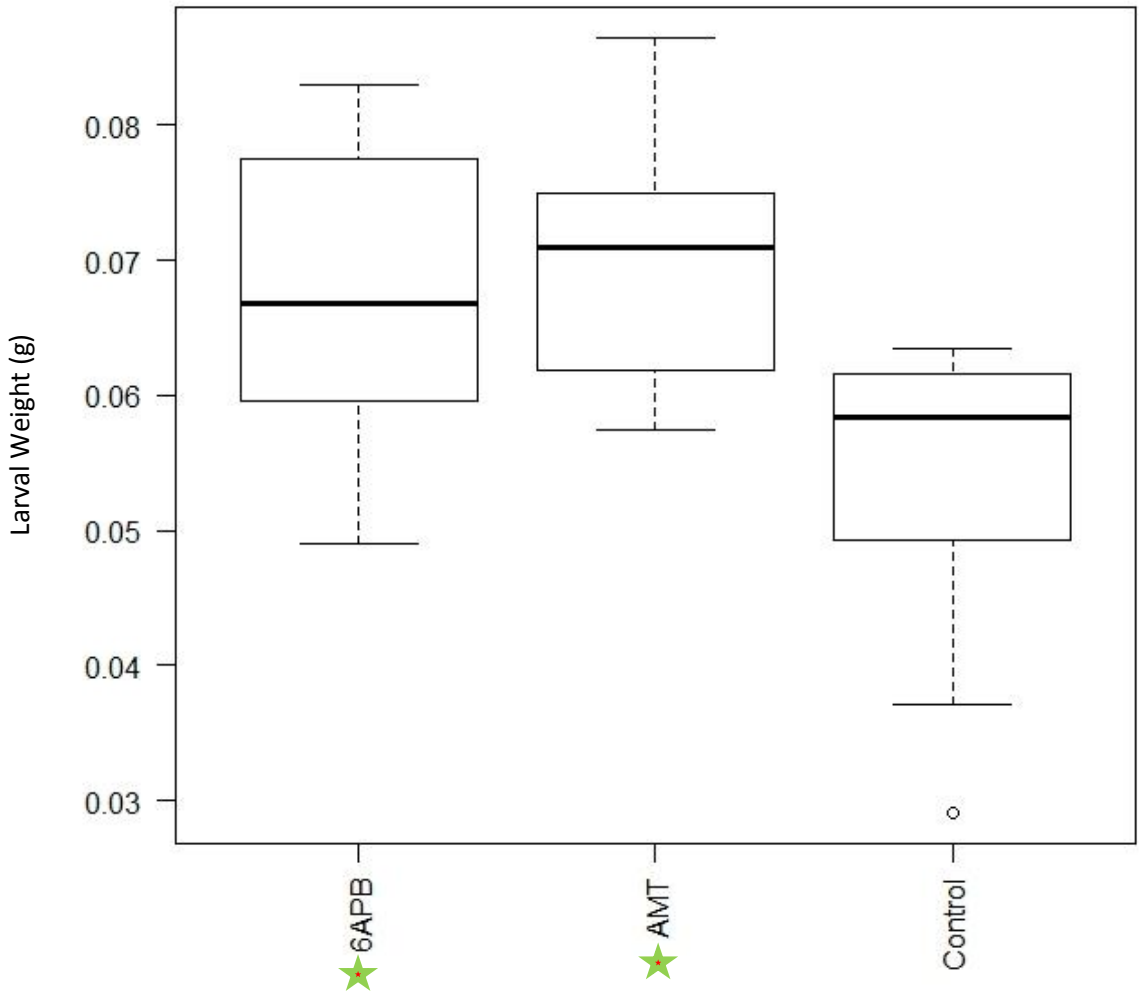


Figure 149: Box plot showing larval weights for day 5 *L.sericata* across selected samples.

Table 28: The minimum, maximum and mean lengths and weights (including standard deviation) on day 5 of selected drugs for *L.sericata.*

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	17.41	21.02	19.3718	0.9034	4.66
	Weight (mg)	74.8	130.2	103.9	13.8	13.28
AMT	Length (mm)	18.8	22.47	20.4473	0.9373	4.58
	Weight (mg)	91.5	133.7	110.2	10.2	9.26
Control	Length (mm)	16.33	19.31	17.8	0.8753	4.92
	Weight (mg)	72.5	109.8	93	11.1	11.94

L. sericata, Day 5- Discussion

This data is also alike the pattern seen during the *C.vicina* development in the presence of the researched compounds.

L. sericata- Day 6

Larval lengths on day 6 ranged between 16.79 mm and 20.94 mm. Both diets showed measurements larger than those seen in the control and were significant (P<0.001). Larval weights ranged between 0.0898 g and 0.1306 g and showed to be significantly heavier than the control population (P<0.001), (Figure 150, Figure 151, Table 29).

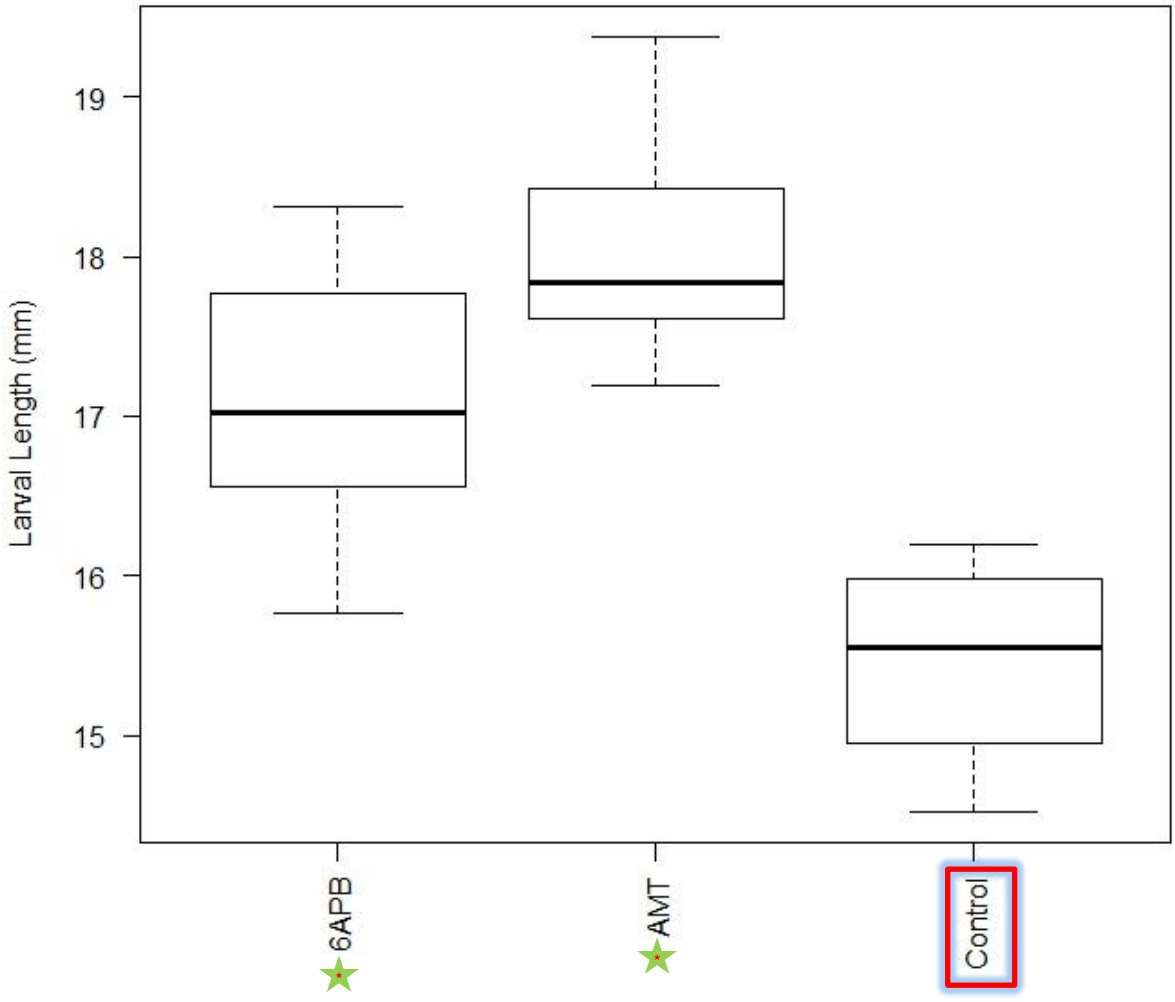


Figure 150: Box plot showing larval lengths for day 6 *L.sericata* across selected samples.

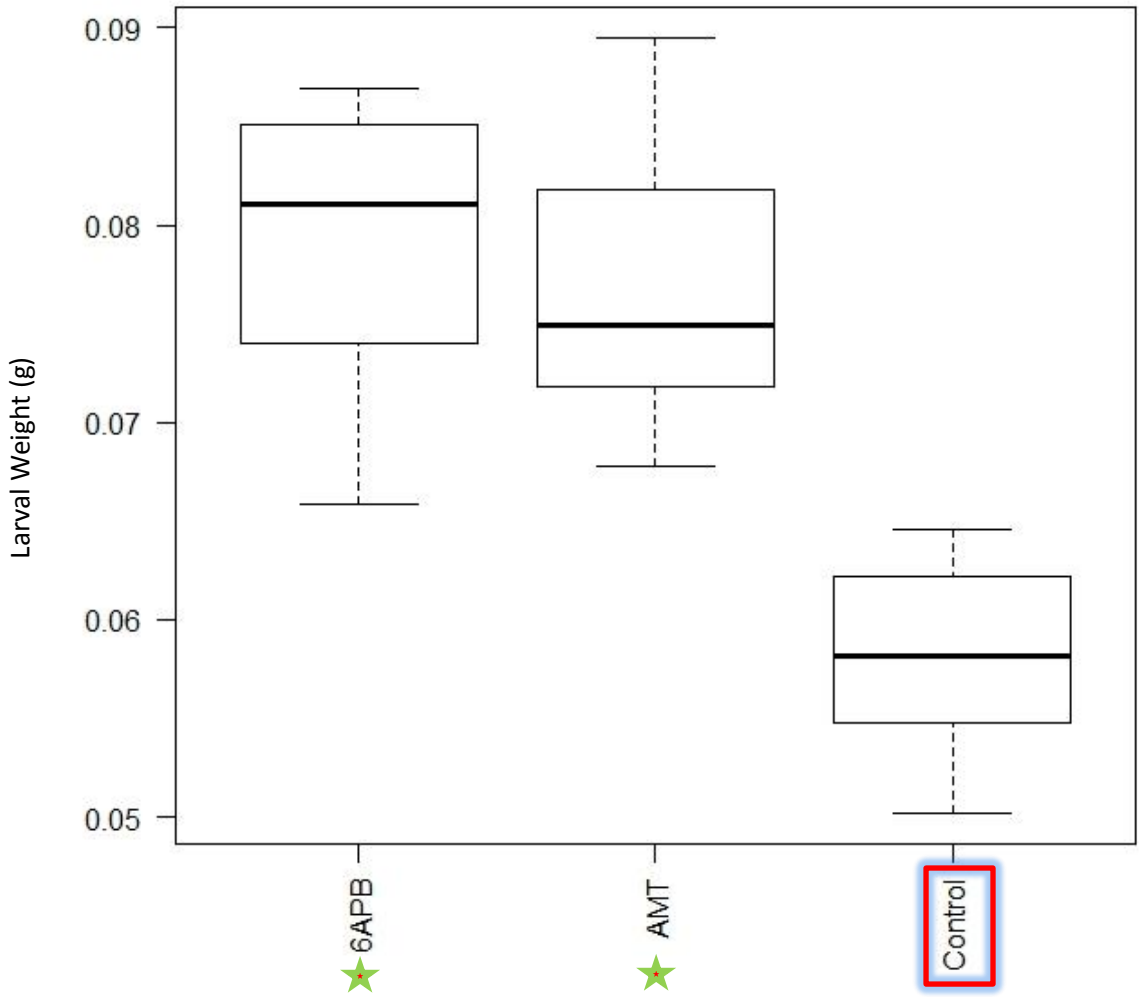


Figure 151: Box plot showing larval weights for day 6 *L.sericata* across selected samples.

Table 29: The minimum, maximum and mean lengths and weights (including standard deviation) on day 6 of selected drugs for *L.sericata*.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	17.88	20.26	19.1455	0.7303	3.81
	Weight (mg)	100.4	130.6	114.9	8.3	7.22
AMT	Length (mm)	19.24	20.94	20.0068	0.4773	2.39
	Weight (mg)	99.7	130.3	116	7.7	6.64
Control	Length (mm)	16.79	18.27	17.539	0.5034	2.87
	Weight (mg)	89.8	105.1	98.9	4.9	4.95

L. sericata, Day 6- Discussion

Comparison with the analysis performed for *C.vicina* shows no differences between the trends found between the diets.

L. sericata- Day 7

Lengths on this sampling day are shown to range between 14.084 mm and 18.186 mm and both AMT and 6APB are shown to be significantly larger than the control population sampled (P<0.001).

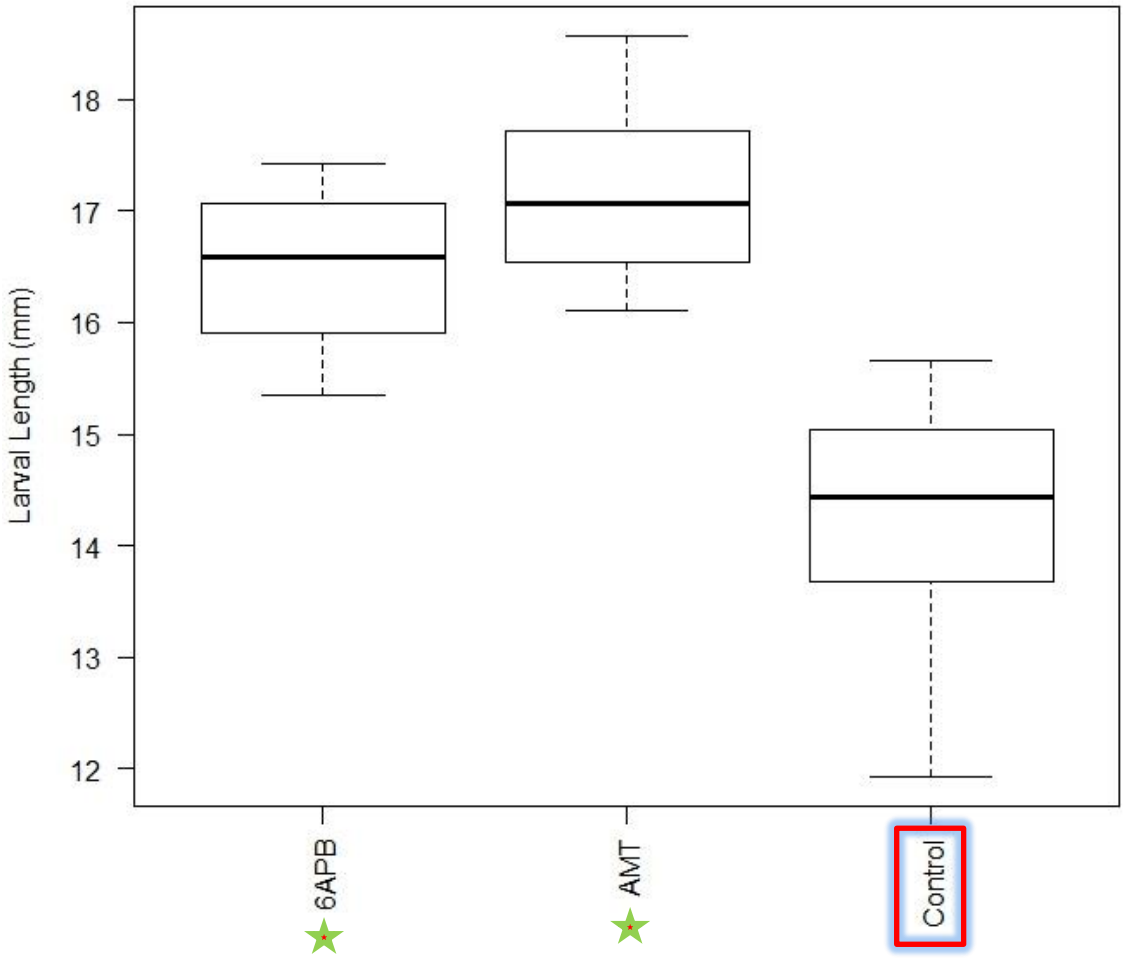


Figure 152: Box plot showing larval lengths for day 7 *L.sericata* across selected samples.

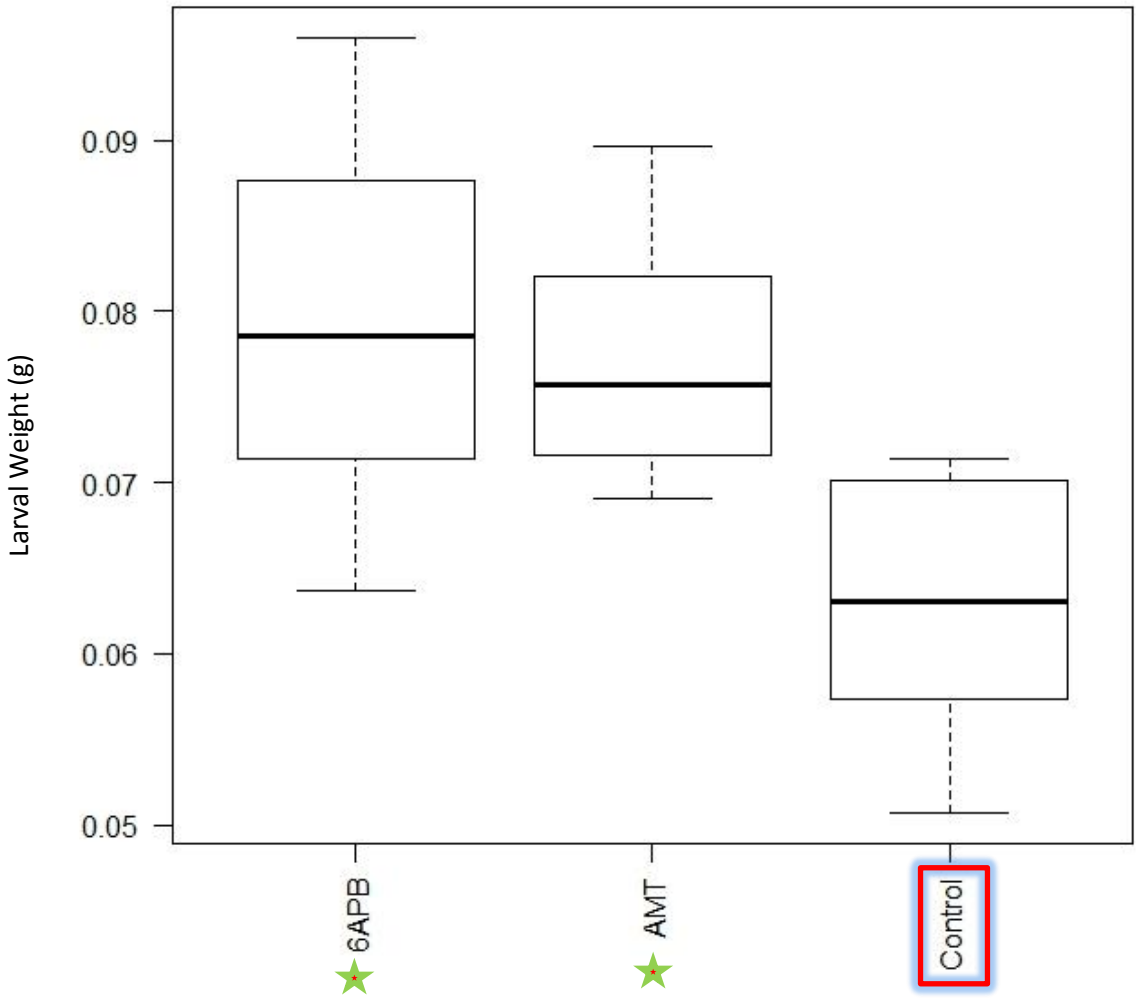


Figure 153: Box plot showing larval weights for day 7 *L.sericata* across selected samples.

Table 30: The minimum, maximum and mean lengths and weights (including standard deviation) on day 7 of selected drugs for *L.sericata*.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	17.344	19.406	18.4913	0.6537	3.54
	Weight (mg)	89.6	127.1	109.3	10.4	9.52
AMT	Length (mm)	18.186	20.546	19.181	0.6929	3.61
	Weight (mg)	93.1	121.7	106.6	7.4	6.94
Control	Length (mm)	14.084	17.606	16.326	0.9349	5.73
	Weight (mg)	77.6	109.4	94.3	9.4	9.97

L. sericata, Day 7- Discussion

Comparison with the analysis performed for *C.vicina* shows no differences between the trends found between the diets.

L. sericata- Day 8

Larval lengths ranged from 14.726 mm to 15.534 mm, only AMT was shown at this stage to be statistically different to the control population (P=0.0161), its growth was slightly accelerated. 6APB was shown to be similar (Figure 154). Weights ranged from 0.0684 g to 0.1204 g with both diets showing a slight increase in weight. (P (6APB) =0.00534, P (AMT) =0.04989), (Figure 155, Table 31).

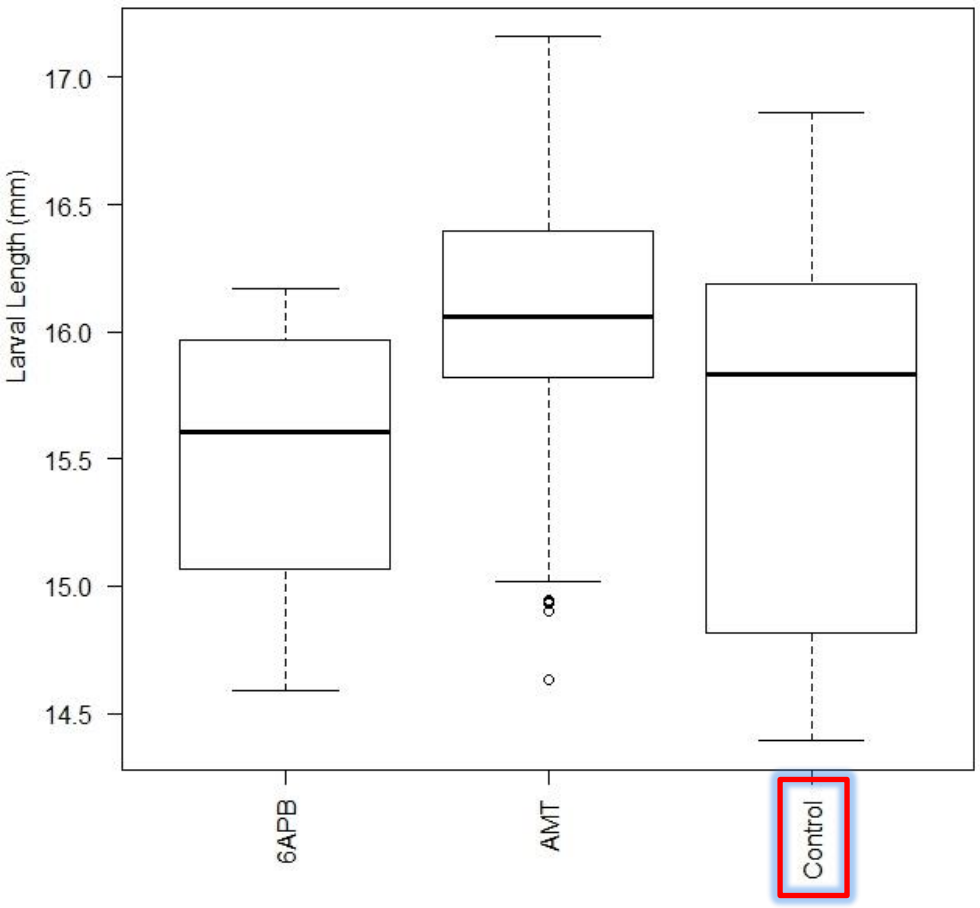


Figure 154: Box plot showing larval lengths for day 8 *L.sericata* across selected samples.

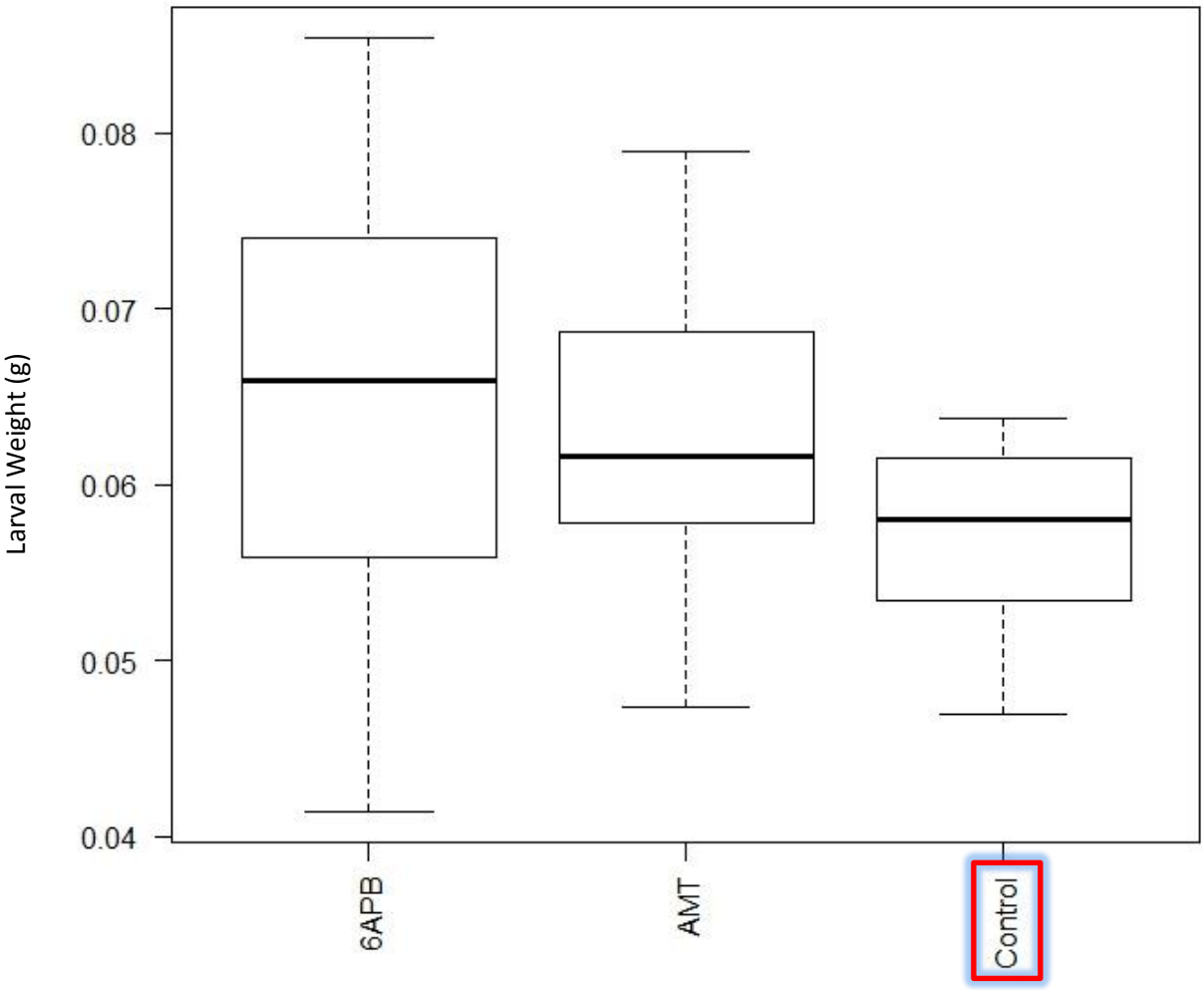


Figure 155: Box plot showing larval weights for day 8 *L.sericata* across selected samples.

Table 31: The minimum, maximum and mean lengths and weights (including standard deviation) on day 8 of selected drugs for *L.sericata*.

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
6APB	Length (mm)	15.534	17.156	16.4655	0.4939	3.00
	Weight (mg)	68.4	120.4	96.1	13	13.53
AMT	Length (mm)	14.726	18.112	16.883	0.895	5.30
	Weight (mg)	73.6	108.5	91.6	8.3	9.06
Control	Length (mm)	15.406	17.764	16.5415	0.796	4.81
	Weight (mg)	74.8	95.8	87.3	5.5	6.30

L. sericata, Day 8- Discussion

This is the only sampling day where differences between the two species are shown. Neither 6APB nor AMT were statistically different to the control in the population of *C.vicina* at day 8 when sampling larval weight. In *L.sericata* measurements, AMT was shown to be slightly longer than the control and 6APB showed an increase in weight compared to the control, where a decrease was actually shown in *C.vicina*. These differences are ever so slight.

L. sericata- Day 9

For both 6APB and AMT populations, at least 50% of the individuals had pupariated by this sampling stage for this species. Length of the ranged from 13.726 mm to 16.584 mm and weights ranged between 0.072 g and 0.0956 g (Figure 156, Figure 157, Table 32).

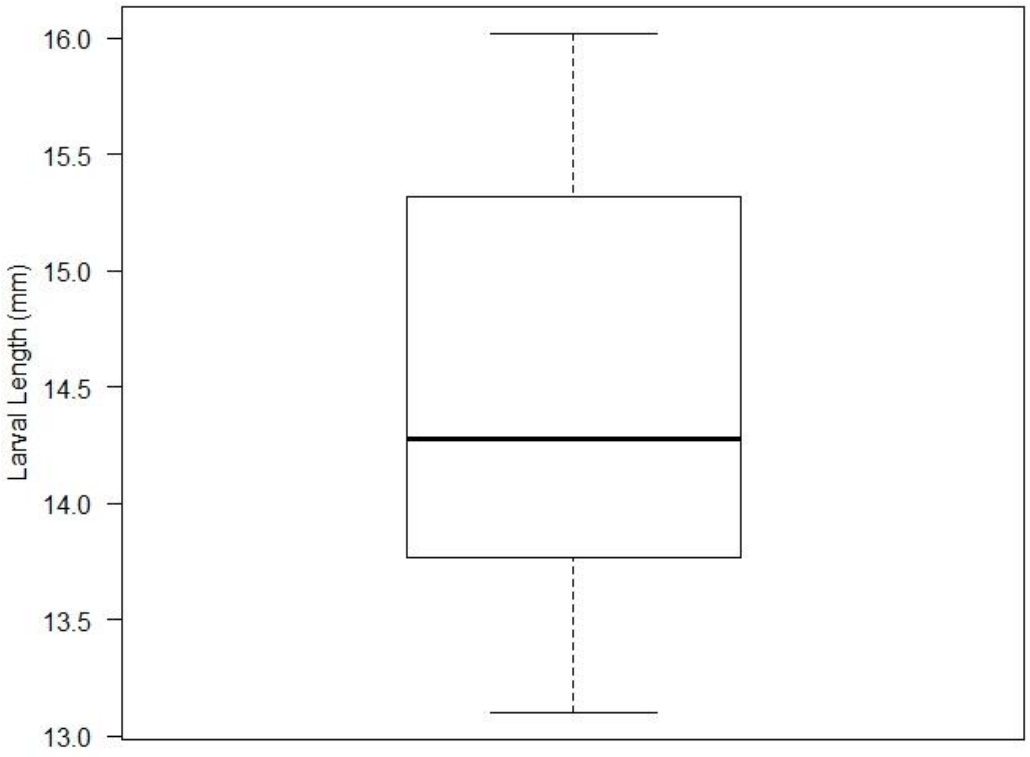


Figure 156: Box plot showing larval length for day 9 *L.sericata* Control.

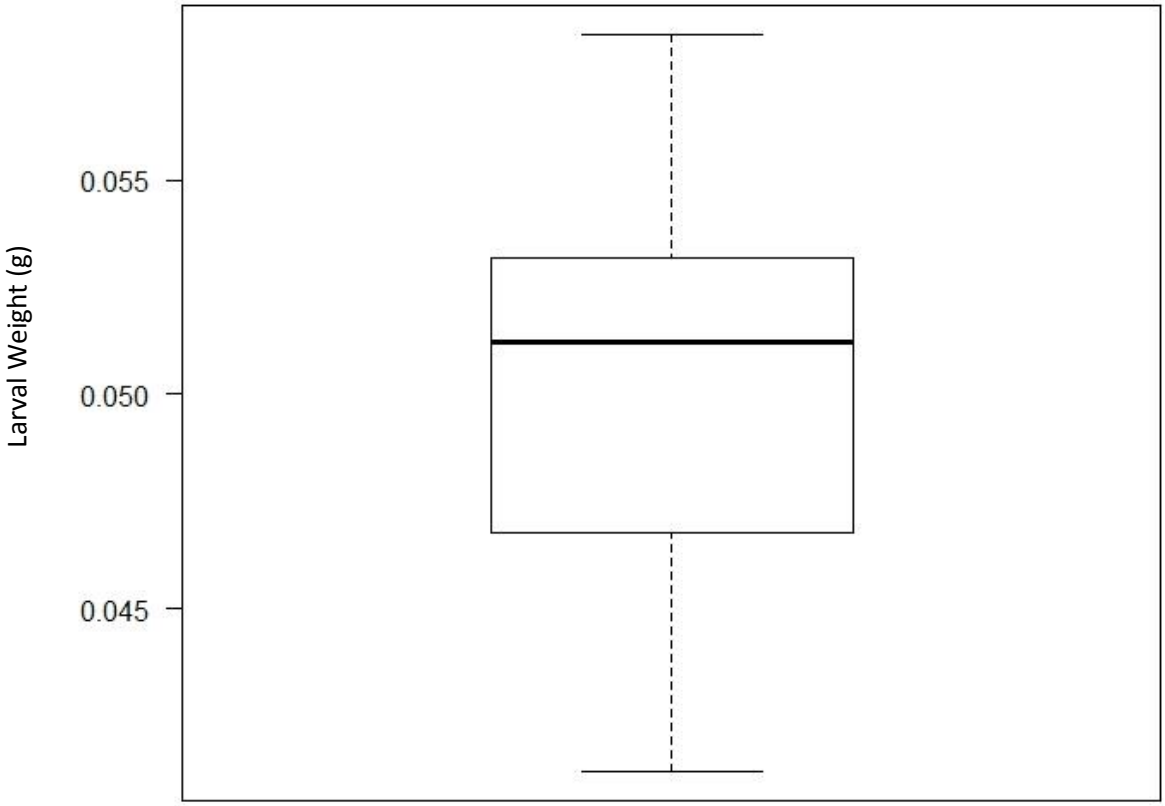


Figure 157: Box plot showing larval weight for day 9 *L.sericata* Control.

Table 32: The minimum, maximum and mean length and weight (including standard deviation) on day 9 Control for *L.sericata.*

Drug		Min	Max	Mean	Standard Deviation	% Standard Deviation
Control	Length (mm)	13.736	16.584	15.1265	0.932	6.16
	Weight (mg)	72	95.6	85.3	5.6	6.57

L. sericata, Day 9- Discussion

Comparison with *C.vicina* showed that both of these drugs had caused early pupariation on day 9 also.

5.2.5.2 Species Discussion

Previous research has suggested that different species may react differently to drugs; this variation also appears within blowfly species of the same family [150]. A study showing the effects of Morphine on the developmental rate of *L.sericata* showed that the higher concentration delayed development [148], this result is not the same as reported for Sarcophagidae [153]. It is possible that certain species show less reaction than others [122], but also that species may develop to enable resistance to specific drugs they have come into contact with and their effects. It must also be considered that species could potentially differ in their reaction to drug presence due to their elimination and accumulation processes.

The original species used within this investigation was *C.vicina*, two drugs at two dosages were selected to use comparatively with *L.sericata*. These drugs showed very limited developmental differences between the two forensically important species. It is not possible to claim that all the developmental changes observed within *C.vicina* would be replicated in *L.sericata* without further investigation.

Principle component analysis was carried out at day 1 and day 6 so show the developmental differences between the two species at these time points. 100% of the data variance is shown in this plot using PC 1 and 2. The results are shown in Figure 158 and Figure 159. No clear distinction between sampling groups can be made at day 1. By day 6 however, the different sample groups are distinctive. Notably the same pattern is shown for both species, suggesting the affects upon development are the same although slightly adjusted as species measurements differ slightly.

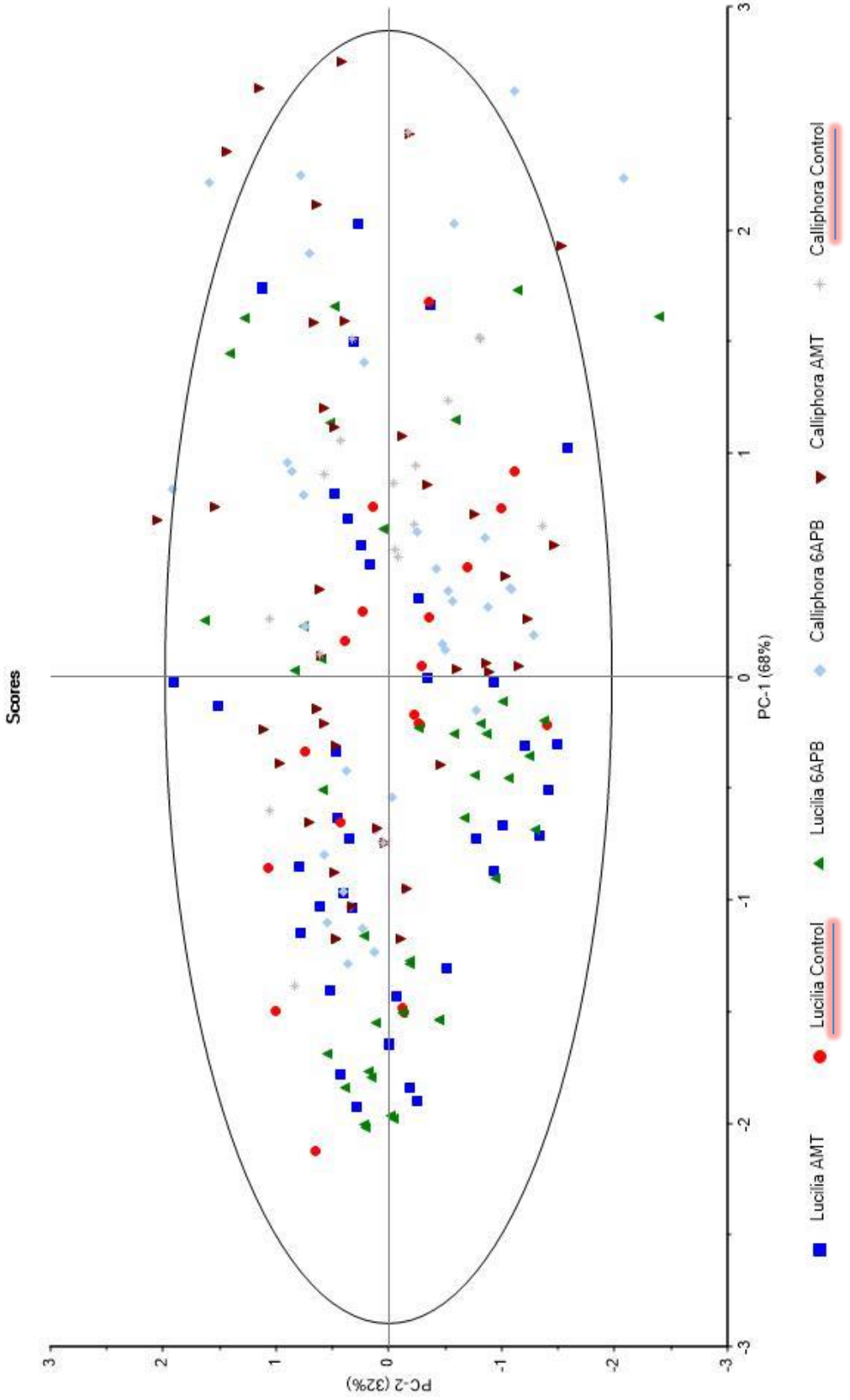


Figure 158: PCA plot showing measurements of selected drug diets for *L.sericata* on day 1.

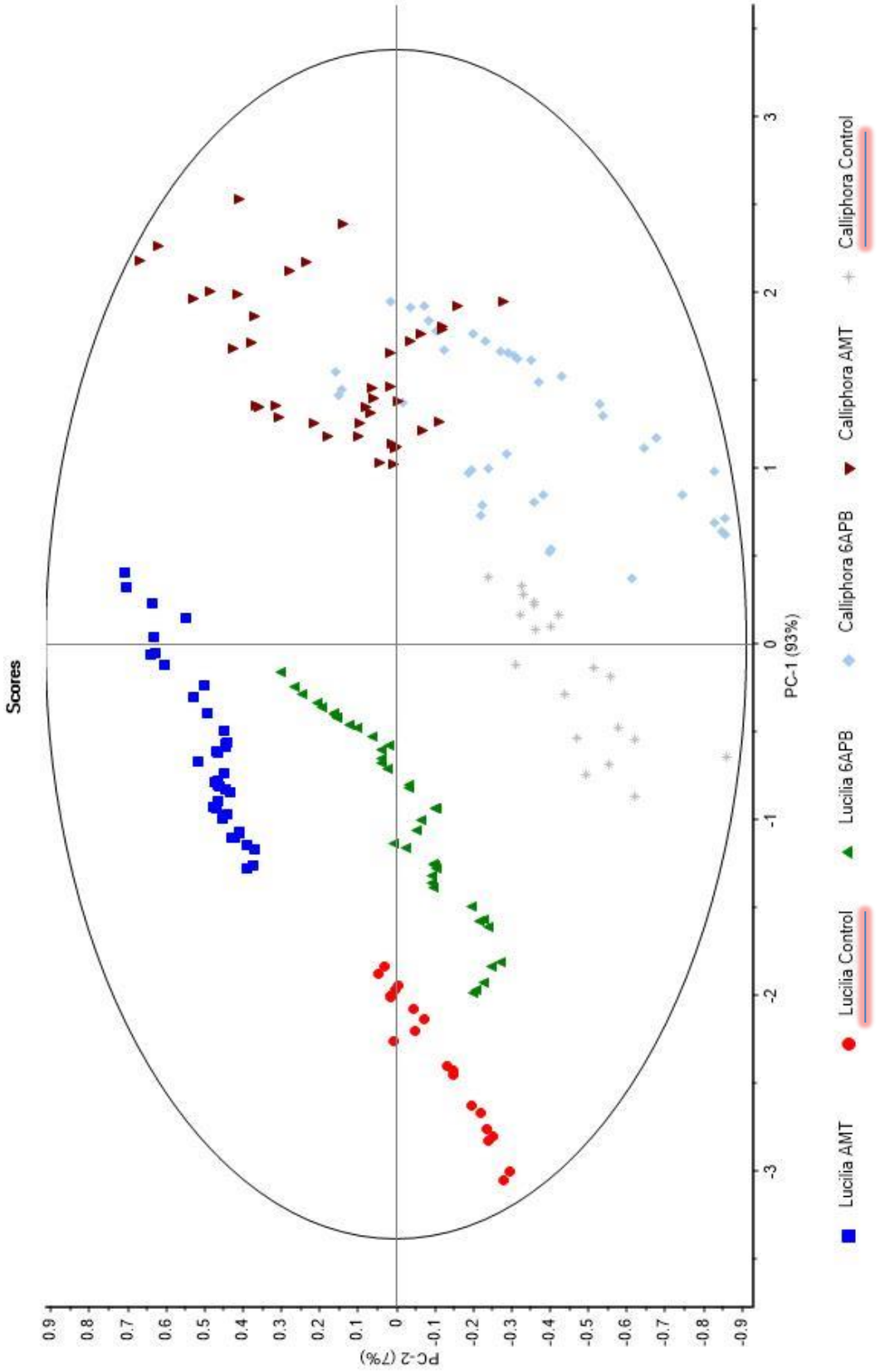


Figure 159: PCA plot showing measurements of selected drug diets for *L.sericata* compared with *C.vicina* on day 6.

5.2.6 Comparison of a NPS and its 'illegal' version

It is suggested that 6APB was originally synthesized to provide a 'legal' alternative to MDA, which is a Class A substance in the United Kingdom. It is part of the amphetamine family and is known for its psychedelic and stimulant effects, it is used as a recreational drug.

It was hypothesized that Novel Psychoactive Substances (NPS) of similar chemical structure to another researched drug would display the same developmental effects. As described in chapter one, a major issue regarding NPS is the unpredictability with composition. As already shown, different drugs and different dosages can have multiple effects on developmental data. It would be useful to determine if drugs of similar chemical structure produced results that were comparable. Shown previously in this chapter, both MDA and 6APB have been researched and added to individual diets at two different concentrations to determine their developmental effects. These have already been analysed per day but a direct comparison will now be made using principle component analysis.

Principle component analysis for day 1 plotted 100% of the data variance. No obvious separation of the data was shown using the PCA plot, suggesting that the data is comparable. This is expected, as clustering was not observed at day 1 due to the insufficient time given at this sampling point for any drug effect to be observed (Figure 160).

Principle component analysis for day 6 is selected for comparison with day 1 as by this point sufficient time for any development acceleration or delay is allowed, it is also

before the data starts to plateau in preparation for pupariation. Data points shown in the PCA in Figure 161 are again not particularly separated. It could be suggested that MDA tends to group at the top of the plot with a higher percentage of points shown here, and 6APB at the bottom but there is overlapping visible.

To determine how tightly clustered these two drug diets are grouped, comparison diets must also be added to the plot. Figure 162 shows data from day 6 again, but this time it included the control data and also data from the AMT diet. It is at this point that the grouping of MDA and 6APB becomes clearer. The data is circled to show suggested clustering. This data is encouraging for the potential use of chemically similar researched drug effects as an accurate comparison for unpredictable NPS.

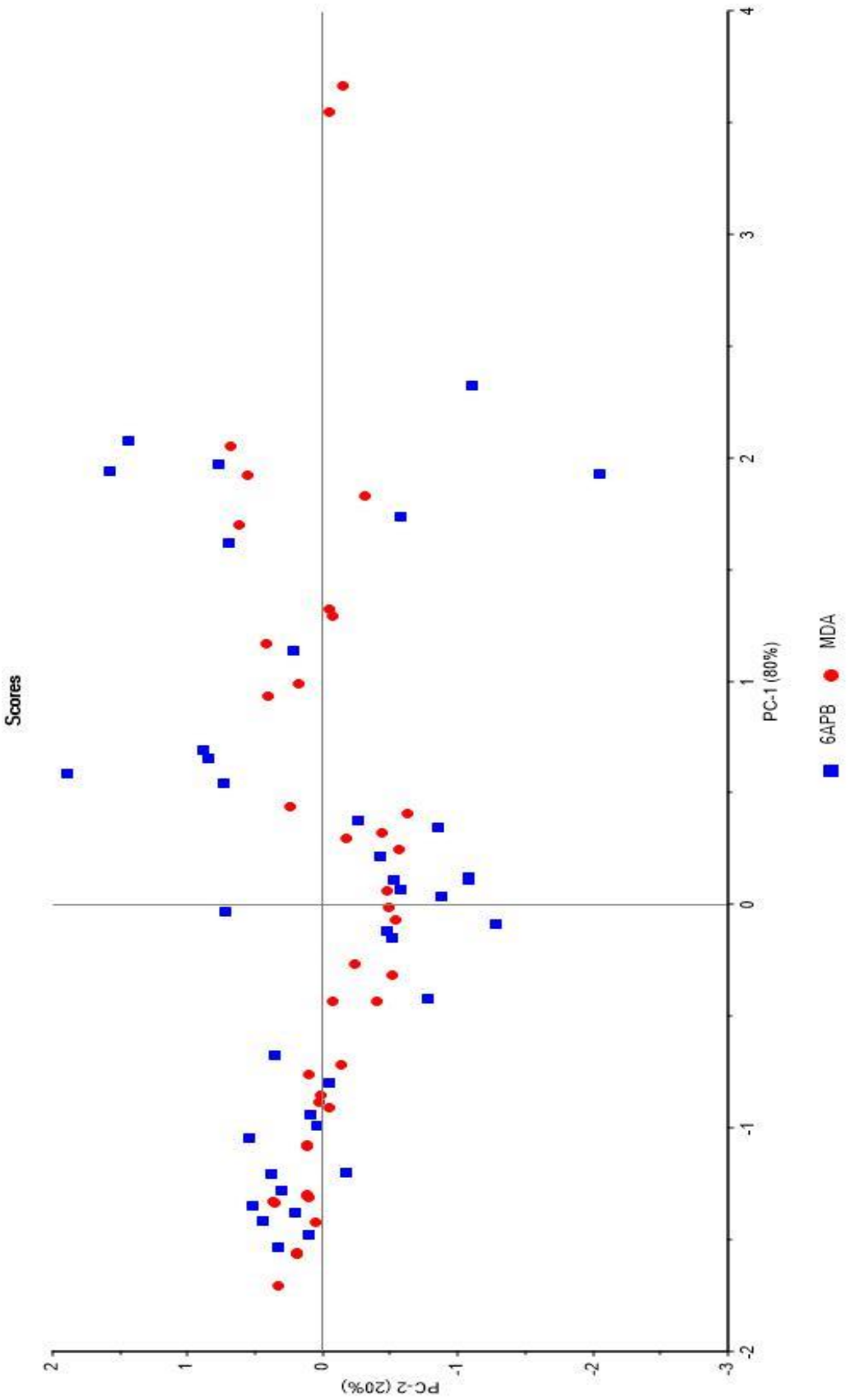


Figure 160: PCA plot showing measurements of MDA and 6APB diets for comparison on day 1.

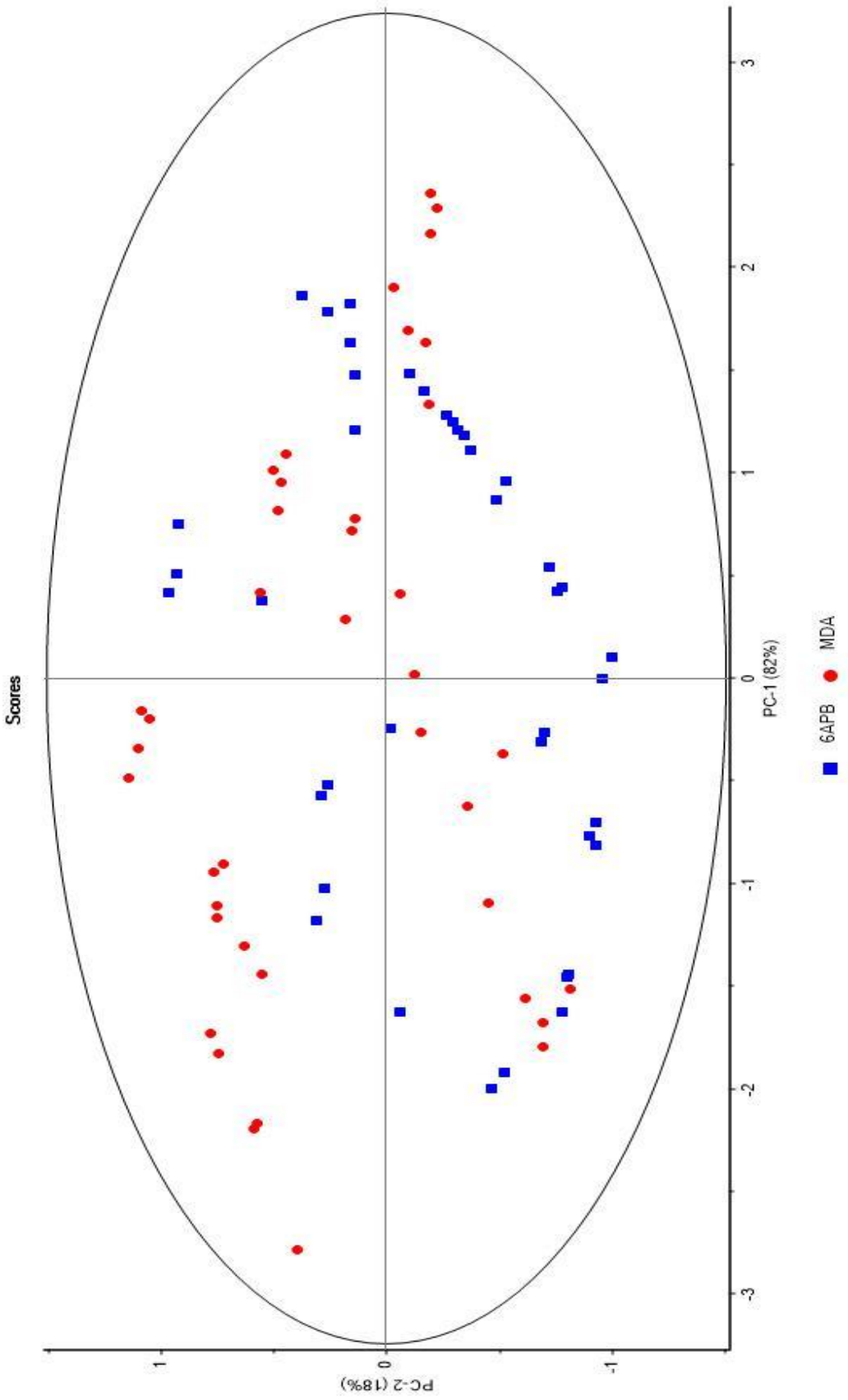


Figure 161: PCA plot showing measurements of MDA and 6APB diets for comparison on day 6.

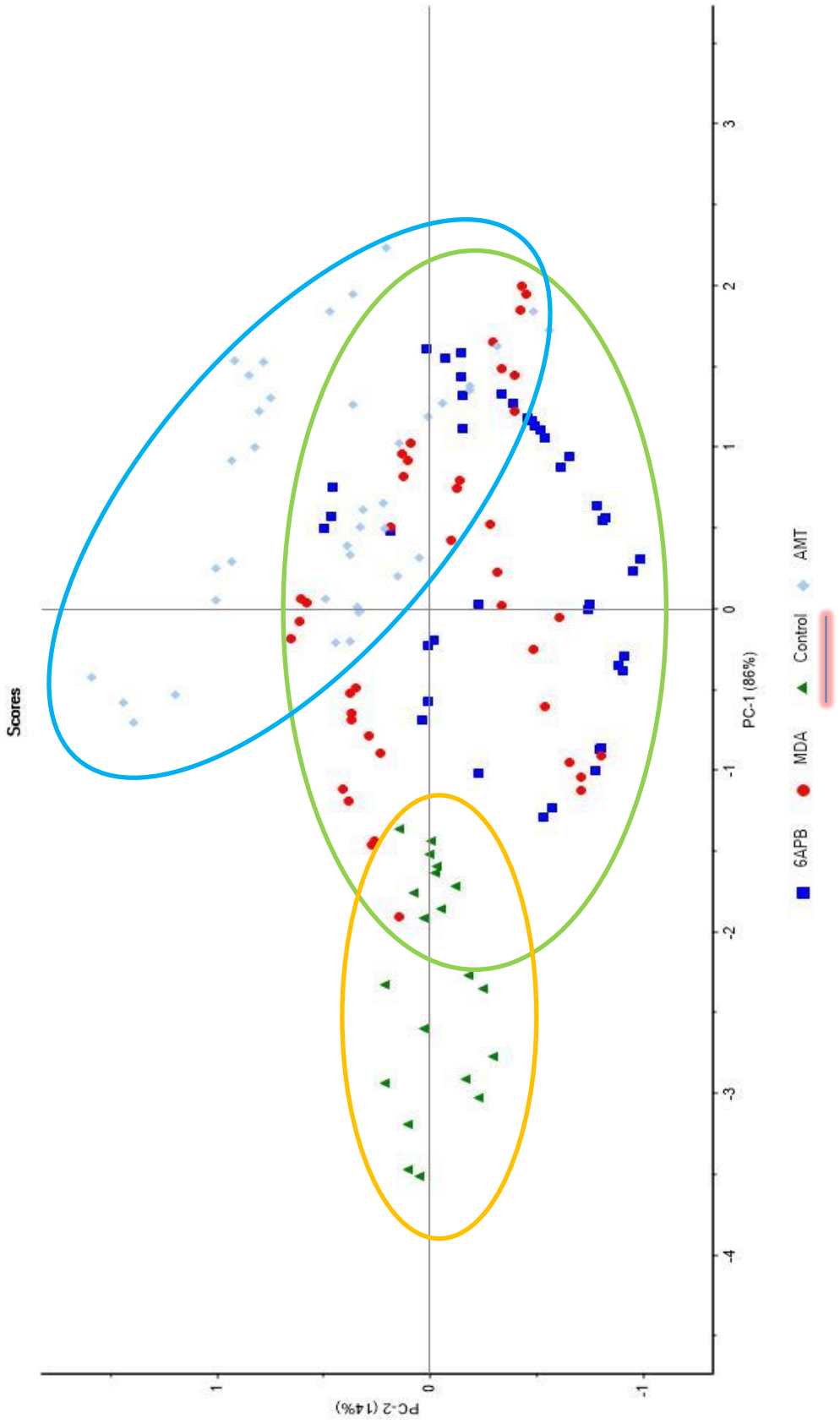


Figure 162: PCA plot showing measurements of MDA and 6APB diets and for comparison Control and AMT (day 6).

Included in Figure 163 is a PCA plot showing 100% of data variance, this plot included data from day 1 to day 9 for the control population alongside 6APB and MDA. Clustering is clearly seen but the amount of data makes it unclear. A breakdown of day 1-day 3 is therefore shown in Figure 164 and day 4 to day 6 in Figure 165.

Day 1 shows all three diets cluster together as there is little data variation. Day 2 clusters again but the control is clearly focussed to one edge of this cluster while MDA and 6APB overlap. Day 3 shows a clearer difference as MDA and 6APB cluster while the control is close by, below. By day 4 the two-drugged diets and the control no longer cluster together, instead the control clusters alone towards the bottom left hand corner of the plot, while MDA and 6APB are above close to Day 5 control data. This suggests that there are more similarities between day 5 control and the drugs at day 4. The drugs at day 5 cluster together in the upper right hand quadrant with day 6 for the drug diets clustering below this. Day 6 control groups more centrally, sometimes overlapping with a number of different groups.

Based on the results shown in this study, using developmental data from MDA for PMI estimation of larvae, which have ingested 6APB, would provide accurate results. It is also clear from these PCA plots that overestimation of larval age is possible using standard developmental data, should the drug presence be ignored.

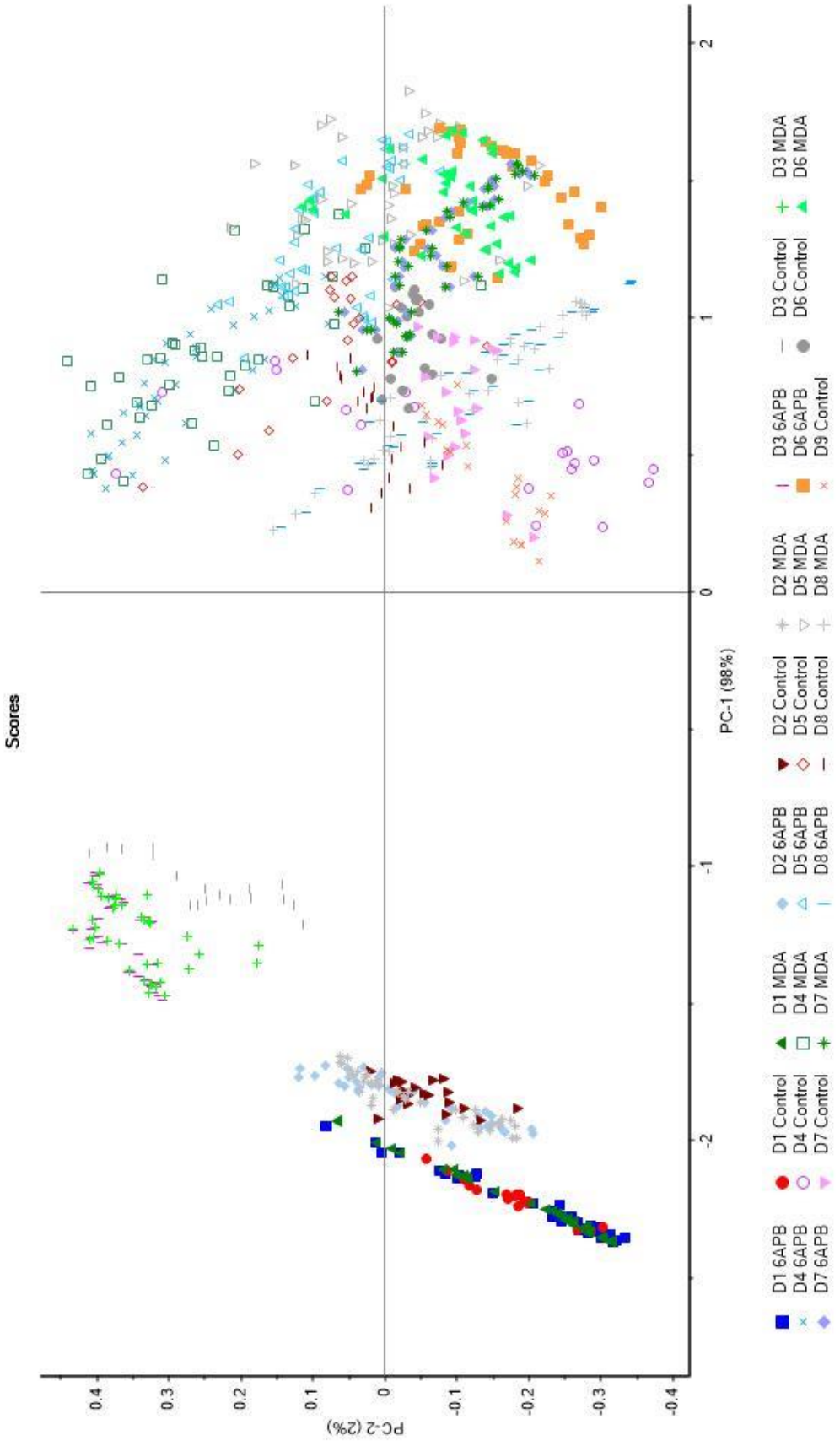


Figure 163: PCA plot showing measurements for Day 1-9 of MDA, 6APB and Control.

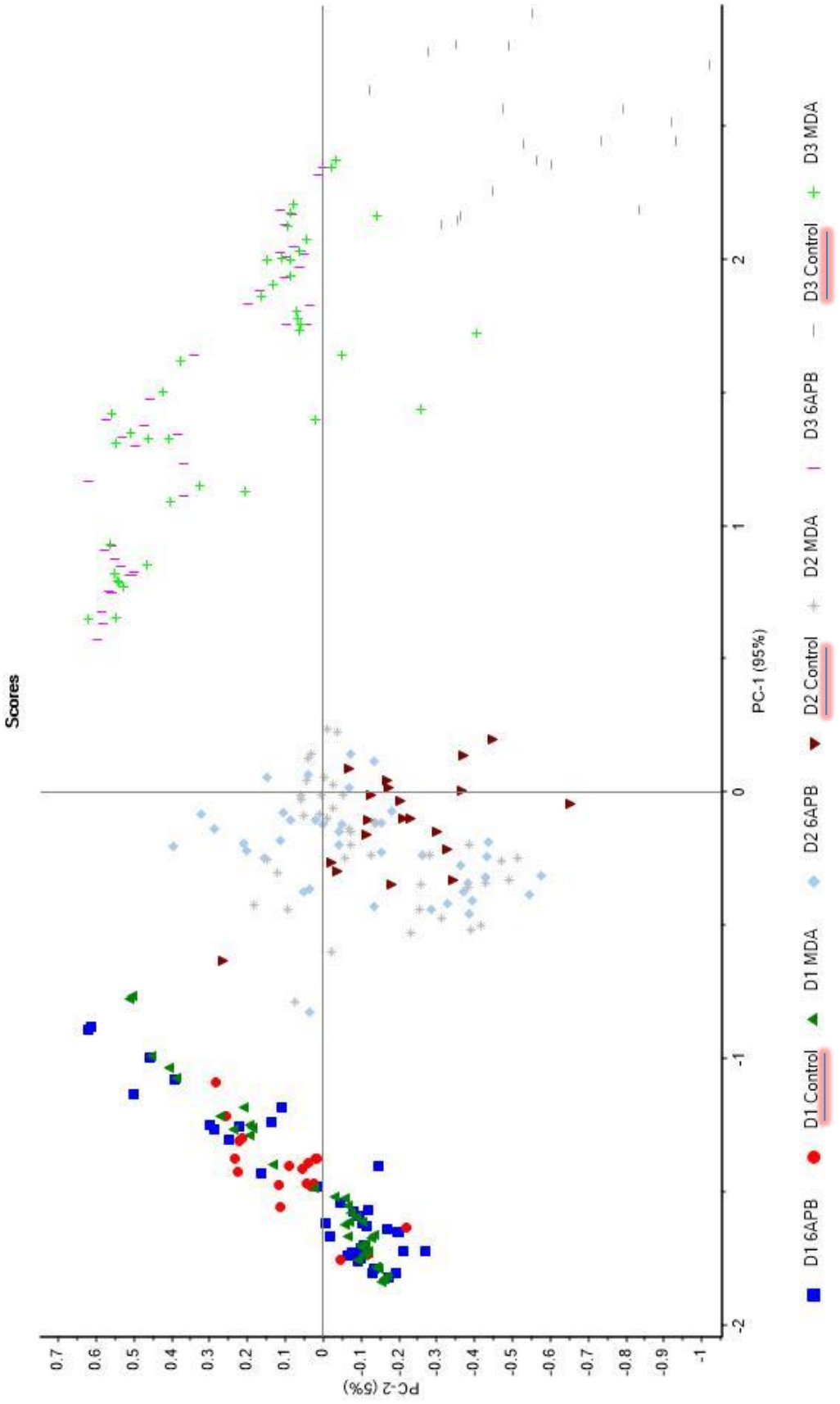


Figure 164: PCA plot of measurements for Day 1-3 of MDA, 6APB and Control.

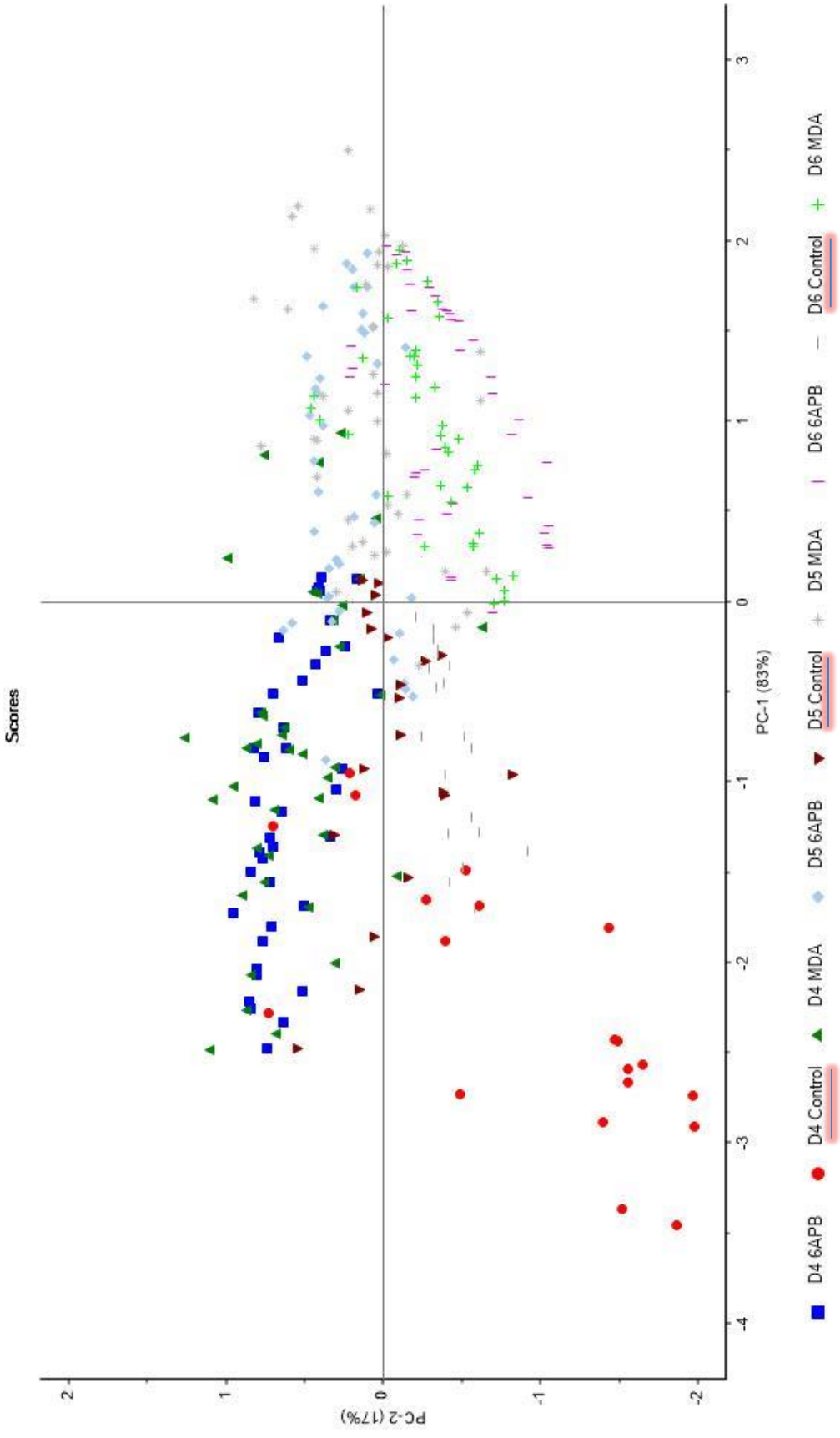


Figure 165: PCA plot of measurements for Day 4-6 of MDA, 6APB and Control.

5.2.7 Effect on viability of adults and oviposition

It is often hypothesized that most drugs ingested by the blowfly are excreted, metabolised or eliminated prior to pupariation [93], [119] so it is not expected that the pupae or adult would be affected by the drug presence. It has however been shown that development is affected by the drug presence and developmental rate and is therefore of interest if the larvae with drugs present during their development complete the puparial stage and emerge as adult blowfly, also if the blowfly, once matured would have the expected ability to lay eggs.

Upon emergence, adults were released into smaller rearing containers where behaviour was monitored. Resulting adults were kept in separate containers for separate drug diets. Milk powder, sugar, water and liver were provided as normal. After giving a period of time to allow maturity of the ovaries, animal tissue was provided to determine the ability of these blowfly populations reared in the presence of drugs, to lay eggs.

Data from this section is purely qualitative as no steps have been taken to quantify these results.

Figure 166: Photo showing setup of induvial cages for monitoring behavioural changes in previously drugged adult blowfly and oviposition investigation.

Each population of larvae had roughly an equivalent number of individuals remaining. This is because a specific number of larvae were used in each experiment, and this was not allowed to vary significantly, so not to introduce experimental error or another area of experimental difference. The same numbers of larvae were sampled from each population, as set out in chapter 3. In addition, emergence from the puparial cases did not appear to differ drastically between diets and the control, with approximately 75% of the individuals emerging successfully across all diets. Survivorship was large enough to finish the experiments and have sufficient individuals remaining to check fertility. Some sample populations were briefly checked and appeared to have an unbiased sex ratio.

The behaviour of the adult blowfly was observed to ensure no obvious differences were present. Behaviour noted was predominantly a lack of unusual behaviour as opposed to any distinguishing behaviour. All populations appeared slow and docile at first but soon

activity increased with the unfolding of the wings. All appeared interested in the provided nutrition and normal flying was observed. This behaviour suggests that drugs are no longer present within the system of the blowfly and/or it is not affecting them in a visible manner.

Often at pupariation, the drug is no longer present within the blowfly due to elimination and metabolism. This could also be the reason why pupal survival rate does not appear to be affected in this study. Pupariation was shown to occur earlier in a number of populations, this has been highlighted in previous studies also; cocaine was shown to encourage earlier pupariation with higher doses showing even further developmental acceleration [153].

Upon presenting the adult blowfly with a second amount of animal tissue a few days after the first, a large amount of interest was shown. The tissue was checked after 3 hours to determine if eggs had been laid. In every case, a number of egg clusters were observed. These were allowed to rear through to first instar to ensure that the eggs were also viable.

Some physiological aspects are not well investigated according to literature [133]. This includes sex ratio and mortality rates. No effect on sex ratio was measured within this study and Mortality rate also did not appear to be affected by the drugs.

There was also no difference shown in relation to the duration of the pupal stage or the percentage of eclosion, this agrees with other studies [153]. Adult flies eclosed and then reproduced successfully within the lab environment indicating that their fecundity had not been affected due to the drug presence within their diets.

5.2.8 Overall Conclusion

This chapter has studied the drug-induced changes on blowfly physiology, and the influence of this on minimum post mortem interval estimation. The developmental changes caused by MDA, 11 NPS and 3 common adulterants and drugs within their own right, in comparison with a control group has been shown for *C.vicina* and this related also to an alternative species, *L.sericata*. For each of these, two dosages were researched; this was achieved using an artificial diet to facilitate the ingestion of the compounds. It must be kept in mind during analysis of this data that the drugs were not metabolised and this would have altered the availability of these drugs and their metabolite for the larvae and this could also have the potential to alter development.

All except the paracetamol diet caused acceleration of development causing a PMI over estimation of up to 48 hours. Paracetamol slowed development and could cause an underestimation of PMI by up to 48 hours, when compared to the developmental rates from the control population. Similarly, One study showed a 24 hour underestimation of PMI would be possible if the drug effect of Morphine was not taken into account [148].

Two dosages were chosen, one considered a high dose, one low, but both realistic. Dose was shown to be of high importance and must be considered. For some drugs, higher doses accelerated growth but not as much as the lower concentration. It is suspected that this is due to the diet itself seeming unpalatable to the blowfly. For other drugs, a higher dose accelerated growth further. A higher dose for paracetamol decreased the developmental rate further. For the drugs tested on both species, 6APB and AMT,

developmental rates followed the same trend seen previously, including the effects of dosage.

The effect of Novel Psychoactive Substances on developmental rate of both *C.vicina* and *L.sericata* showed significant differences. The suspected age of insect samples found at a crime scene are used to estimate the minimum PMI as explained in chapter one. Temperature is one element accounted for during this calculation, it would however lead to incorrect estimations if the presence of a drug is overlooked [309]. Ignoring interfering factors such as drug presence and dose could lead to either an over or underestimation. The substances chosen for use recreationally will differ area to area dependent upon availability, culture, lifestyle and policing [150]. Both the study presented here and previous research has shown the implications of these substances on the development of the blowfly, it is important therefore that the effects of common substances of misuse on blowfly species of forensic importance is known. Novel Psychoactive Substances must be considered due to their widespread use and the unpredictable nature of their composition.

Previous studies have shown that development rate in response to drugs can increase, decrease or in some scenarios, remain the same [121], [128], [147], [148], [150]. Differences were observed in all cases when adding NPS and common adulterants into the food source of the blowfly populations.

The effect of the drug on behaviour, including feeding behaviour, must be acknowledged with as much weight as the changes shown in development, although these are more difficult to quantify.

Without the use of an animal model where the drug is administered prior to death, attention must be paid to the non-metabolism of the drug of interest. Metabolism will alter the availability of both the drug and its metabolites. The drugs may however be metabolised differently in humans and animals. Drugs and their metabolites both affect blowfly development when they are found to be present in colonized cadavers [114]. The usefulness of results must be determined prior to use on real cases in relation to differences in protocol. Areas of consideration include metabolism of the selected drug and its stability, alongside time for completed analysis as this may all influence the results [310]. A further consideration is determination of whether or not the conditions associated with a cadaver would be affected by the presence of drugs, other than the effects caused by a change in insect behaviour caused by them.

Entomotoxicological data is very useful within forensic investigations, assuming all aspects of variance are considered. This reinforces the need for further investigation of the effect of these drugs at different concentrations on the developmental rate of all forensically important blowfly species.

Sex ratio, pupal survival rate and fecundity all appear unaffected by drug presence in this thesis, likely due to drug metabolism and elimination.

6.0 Introduction

As explained previously in Chapter 1, estimation of minimum PMI can be made using insect samples found at the crime scene. Development data specific to the crime scene scenario is required however, and variables that affect larval growth are not always considered, introducing inaccuracies to the estimation. The analysis of the insect cuticle focussed upon hydrocarbons has already shown positive results for taxonomic and aging purposes [187], [221], [222], [232], [234], [239], [311]–[314], and are found within the waxy outer layer of every insect at all life stages, a link between the development of blowfly larvae and cuticular hydrocarbon profile has been revealed. Due to this link between hydrocarbons and aging combined with the stability of hydrocarbons, It is hypothesized that cuticular hydrocarbon analysis may provide a method to estimate PMImin, which is not affected by drug presence. Cuticular hydrocarbon analysis has already been shown in Chapter 4 to be complimentary with the pre-existing sample collection, killing and storage techniques.

Results shown in chapter 5 described the significant developmental differences caused by commonly abused substances, this experiment aimed to determine the effect of these substances on cuticular hydrocarbons. Larval samples were taken daily from those reared on drugged artificial diets, these were extracted as defined in Chapter 3 and analysed using GC-MS. Cuticular hydrocarbons were identified and comparisons between the different populations were made, at this stage cuticular hydrocarbons were not quantified due to time constraints.

6.1 Aims and objectives

The aim of this experimental section was to determine the effect of eleven selected NPS, 3 common adulterants and 1 illegal comparison on the cuticular hydrocarbon profiles of forensically important blowfly species *C.vicina*. Significant developmental differences have previously been found and are presented in chapter 5, it is hypothesised that the drug presence will not encourage change within the cuticle of the insect and this data could be used to provide an accurate estimation of PMI.

Collection of data is briefly detailed below, including statistical analysis; more details can be found in Chapter 3. Results are presented here per sampling day and the findings discussed.

6.2 Methods

Larvae were extracted once daily from emergence of the egg until a minimum of 50% had pupariated. On each day, ten replicate samples were taken per treatment. This generally was a maximum of ten days. In certain cases, the drugs involved in the trial caused early pupariation. The numbers of larvae corresponding to the experimental day were first killed by submerging them in hot water, as explained in chapter three. Developmental data were noted, and each sample was then placed into a labelled vial and fully submerged in hexane. This was then left for ten to fifteen minutes to ensure sufficient hydrocarbons were extracted into the solvent. To avoid contamination the extracts were then run through a silica column.

The oven temperature for hydrocarbon samples was programmed to be held at 50 ^{o}C for 2 minutes then ramped to 200 ^{o}C at 25 ^{o}C/minute, then from 200 ^{o}C to 260 ^{o}C at 3 ^{o}C/minute and finally from 260 ^{o}C to 320 ^{o}C where it was held for 2minutes. Agilent Chemstation software was used to analyse the resulting data.

To ensure that data could be analysed simultaneously and reduce drift, a retention time locked method was set up. This allows close matching of retention time for known compounds. A retention time vs pressure calibration curve was developed using the temperature method explained previously. A number of injections, at defined pressures, of a standard is used to then calculate the retention time at predefined inlet pressures. The method used within the Chemstation software defines the pressure as: 1) the nominal pressure 2) nominal - 20% 3) nominal -10% 4) nominal +10% 5) nominal +20%. The method was 'locked' prior to GC-MS analysis and the drift for the lock compound was observed.

As well as lock compound bromoheptadecane, for the retention time locked method, an internal standard was also utilised to allow possible quantification of results in the future. Docosane was chosen for this as it elutes just before the area of interest but not within.

Statistical software 'Unscrambler' from Camo was used to further analyse the chromatograms. The chromatographic data were exported from Chemstation in CSV (Comma, separated values) format with a selected data range showing retention time against peak height. It was ensured prior to analysis that there was no observed drift between the samples, as this would introduce error. Unscrambler allows alignment of shifted data using its correlation optimization warping pre-processing algorithm.

6.3 Results and Discussion

Predominantly, in the area of interest, it is hydrocarbons, which are observed. It was speculated that the chromatogram range could be narrowed in order to show only the retention times of interest (C18-C33) and then this data could be analysed as a whole profile instead of identification of all the peaks and areas, this would quicken the time taken for analysis. Data from the analysis using this method is shown below.

6.3.1 Analysis of the whole profile without specific hydrocarbon extraction.

The control alongside two further drug treatments was selected to determine the clustering of samples of the same age. Both of these drug treatment populations had previously been shown to cause significant developmental changes after drug ingestion. Samples were plotted to enable visualisation of any pattern resulting. Previous research of *C.vicina* had shown a systematic pattern, clockwise around the plot with increasing age [221].

When observing Figure 167 a scattered plot is shown. There is a large amount of clustering centrally with days 1-3 and days 6-7 appearing together. Previously it had been shown that days 1-2 clustered together due to hydrocarbons specific to only those two days. Day 4 appears above this cluster, day 5 to the left-hand side and day 8 to the right hand side, there is a large amount of overlapping. It is apparent that the trend observed here is variable. Data were further analysed with paired days to determine the level of separation possible using this method.

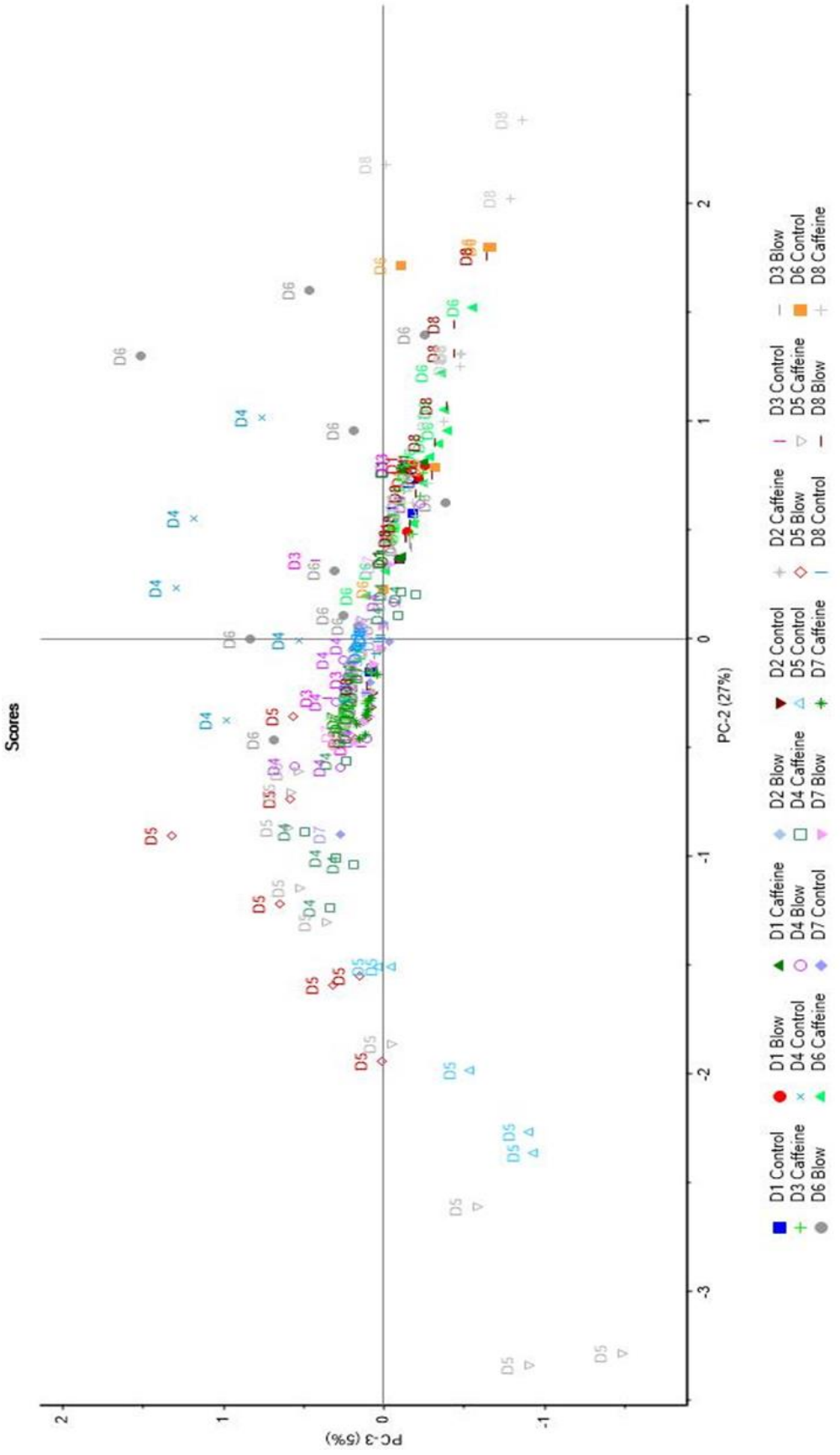


Figure 167: PCA plot of all development days analysed using whole profile.

Days 1-2

Analysis of samples from days 1 and 2 using principle components 1 and 4 (Figure 168), together explaining 86% of the data variance, shows a clear difference between data points is observed. Analysis of the whole profile allows separation of days 1 and 2.

Days 3-4

Analysis of days 3 and 4 (Figure 169) does show a degree of separation, this is not however clearly defined, as had been seen previously with days 1 and 2.

Days 5-6

Analysis of days 5 and 6 (Figure 170) again does show wide clustering of the sampling days, there is a lot more overlapping and data would not be sufficient to differentiate between these two sampling points and allow accurate age estimation.

Days 7-8

Analysis of these two days (Figure 171) shows separation but the clusters are scattered.

Overall

Differences were observed between sampling days, but these would not be sufficient to age larvae accurately. These plots show a large amount of variation and therefore individual peak identification alongside peak area was necessary to determine the cause of this. It is hypothesized that this mass scattering observed is due to noise within the chromatogram, further analysis using extract ion chromatograms could potentially be utilised for future work, further investigation is required.

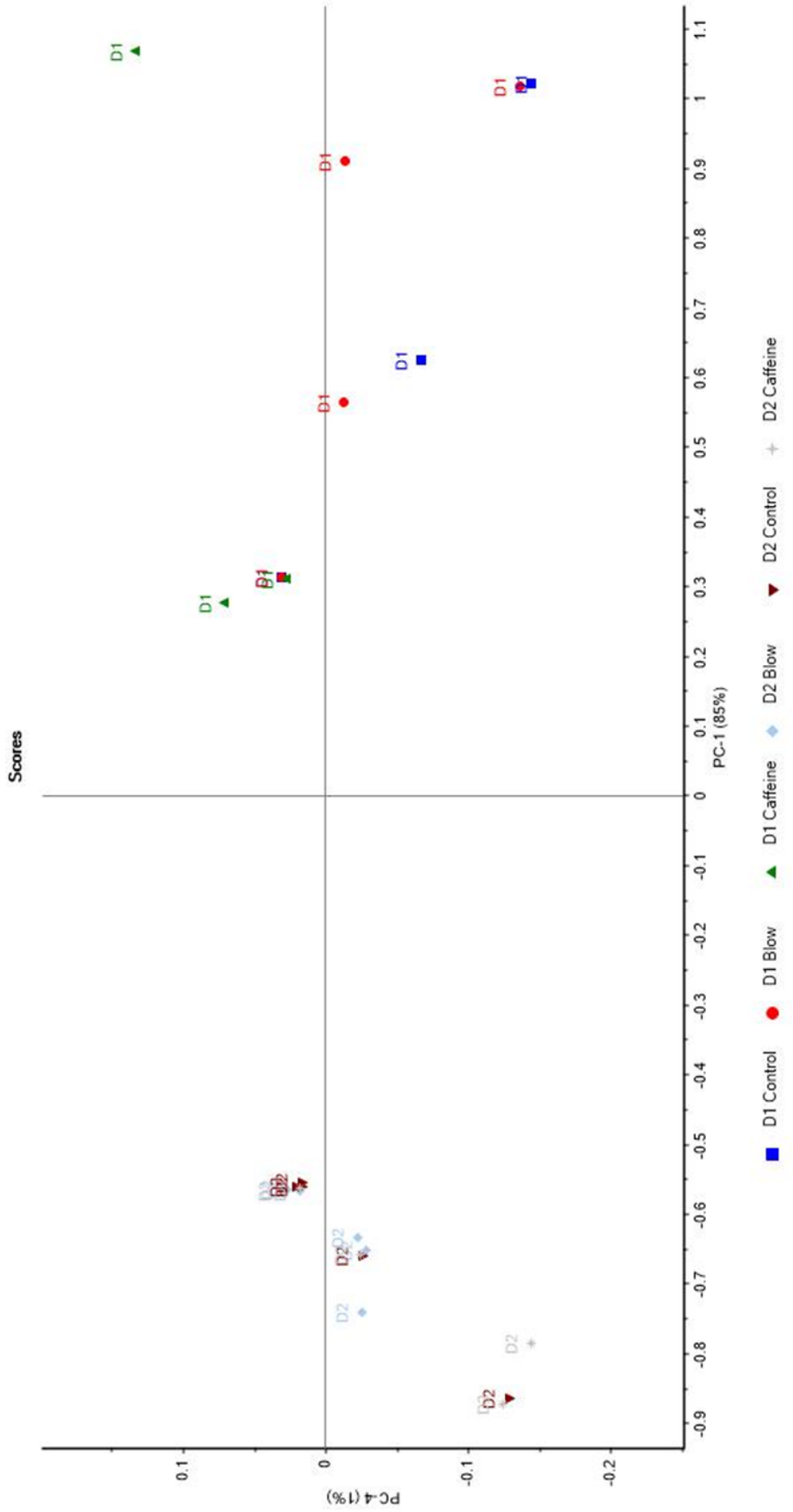


Figure 168: PCA plot of development days 1 and 2 analysed using whole profile.

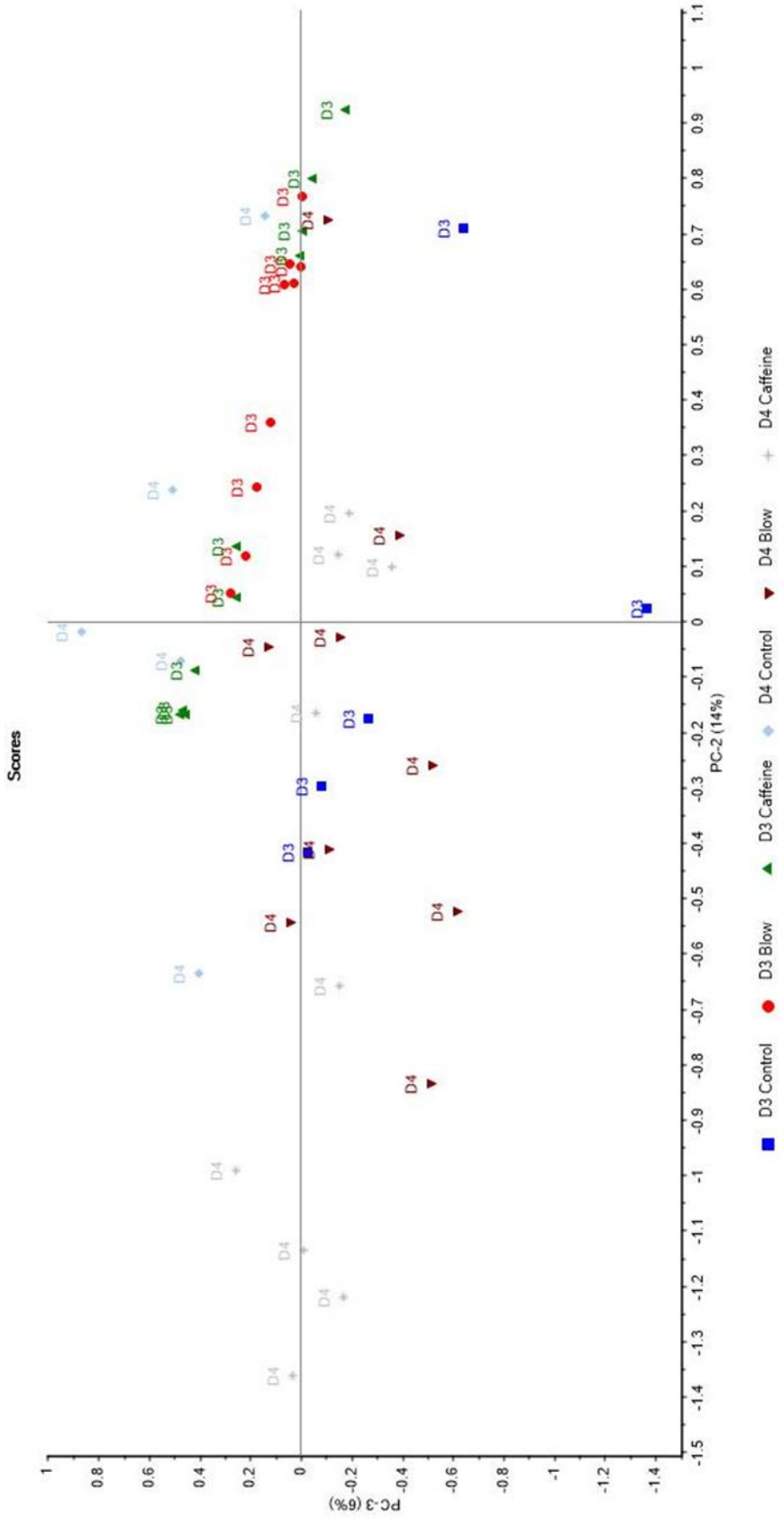


Figure 169 :PCA plot of development days 3 and 4 analysed using whole profile.

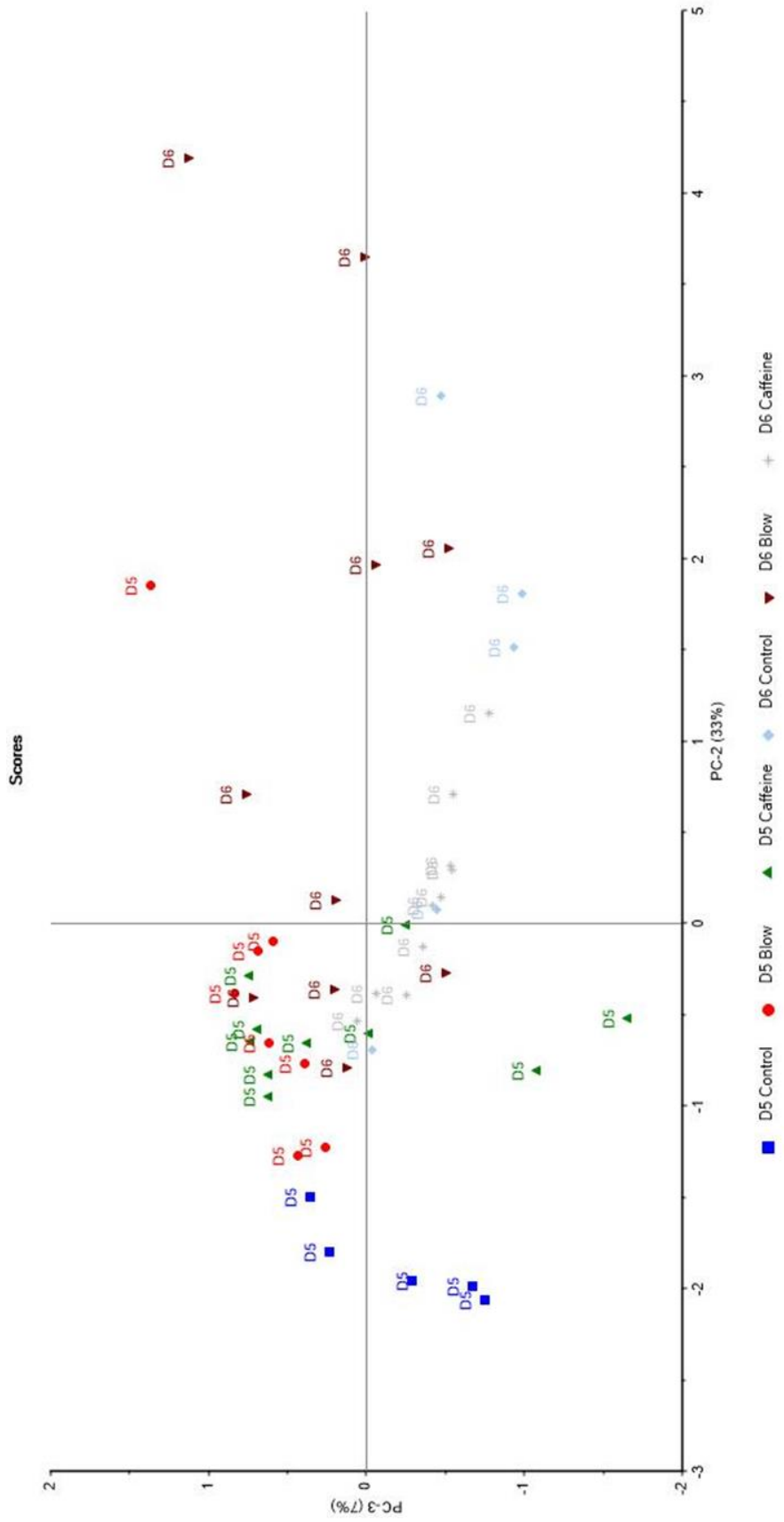


Figure 170: PCA plot of development days 5 and 6 analysed using whole profile.

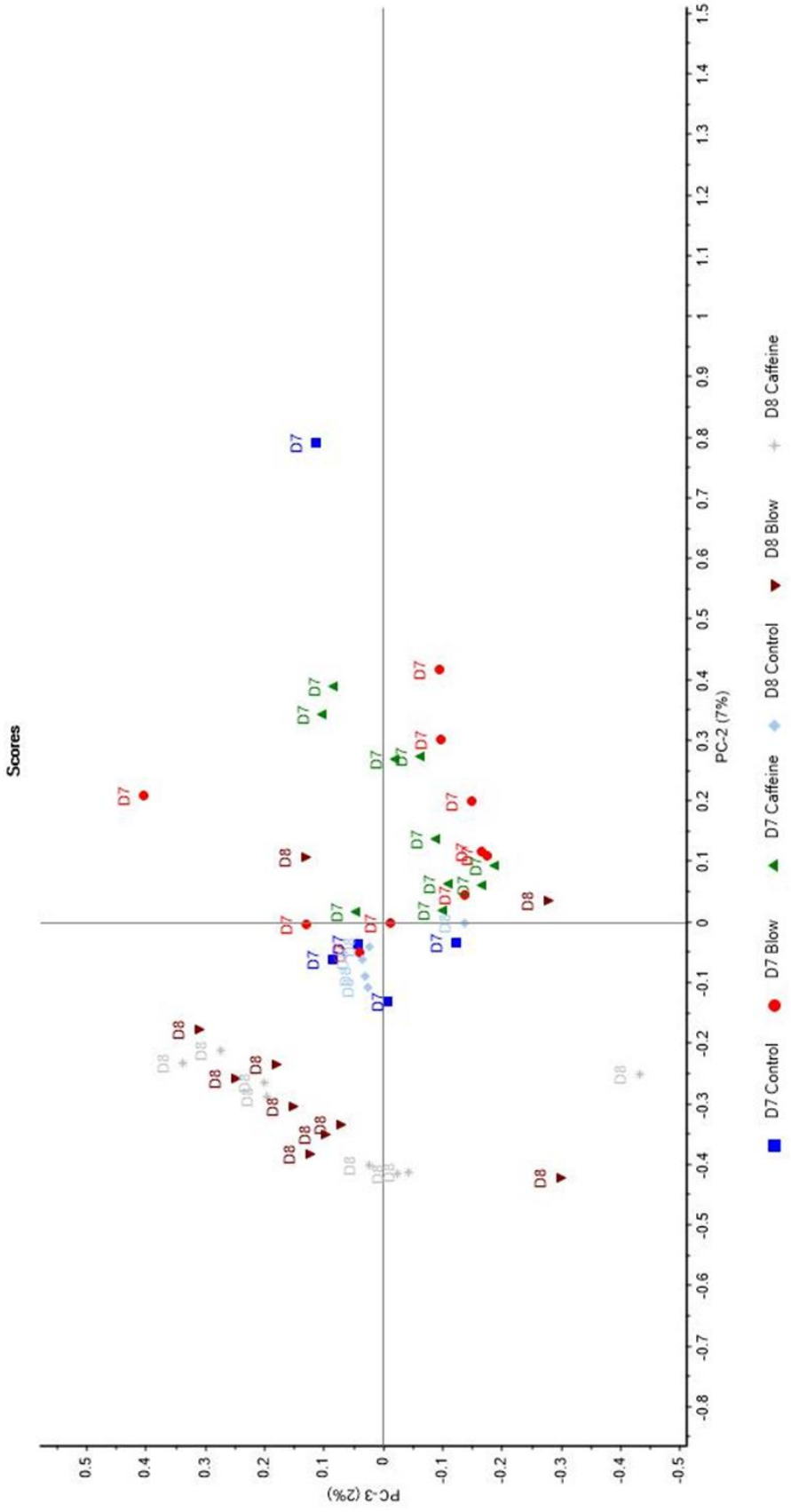


Figure 171: PCA plot of development days 7 and 8 analysed using whole profile.

6.3.2 Analysis following integration and identification of hydrocarbons

Overview

Efforts had been made to discourage retention time drift to enable the accurate analysis of the cuticular hydrocarbon profile. An extract ion chromatogram of the first sample to be analysed on the GC-MS and the last sample are shown in Figure 172 and Figure 174. The retention time of the locking compound can be compared to ensure that the drift was controlled. Ion 137 was chosen, as this was a known fragment ion of the specified locking compound. The mass spectrum of the locking compound with the retention time of the first and last sample analysed, is shown in Figure 173 and Figure 175. No change in retention was observed; Drift was controlled.

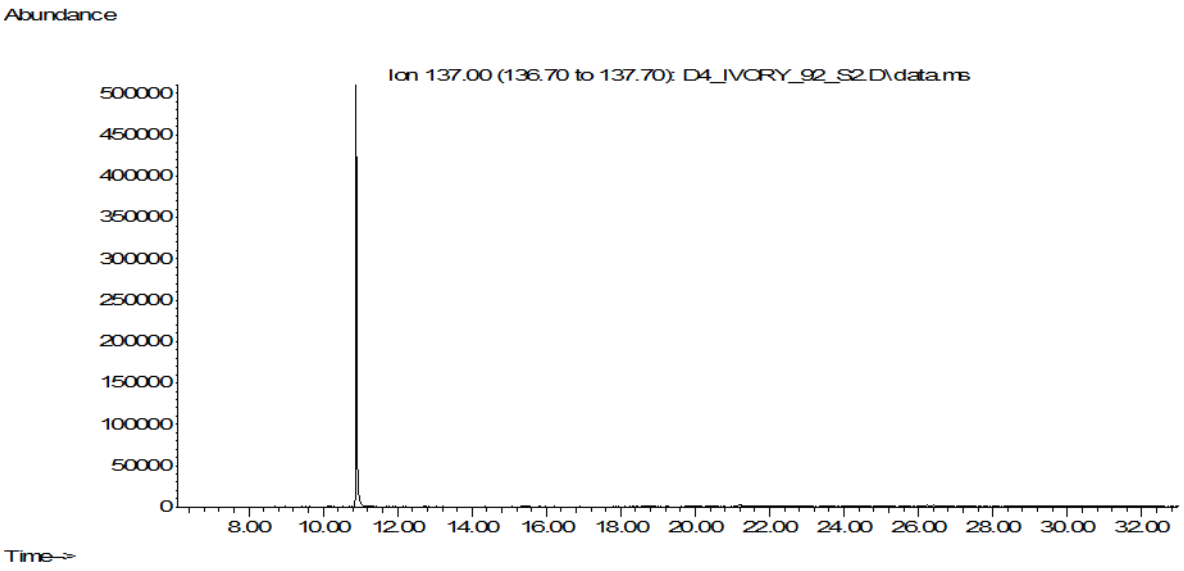


Figure 172: Extract ion chromatogram of the first sample analysed on the GC-MS.

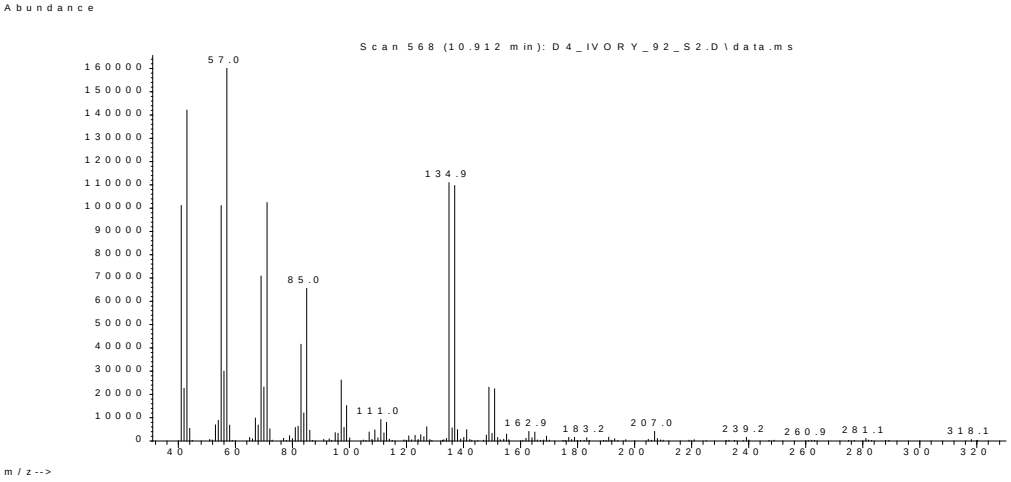


Figure 173: Mass spectrum of Bromoheptadecane within the first analysed sample, with retention time shown.

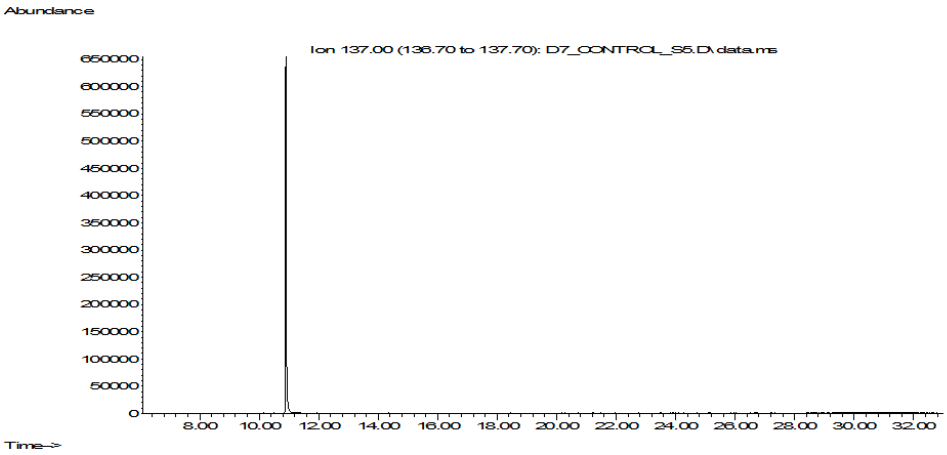


Figure 174 : Extract ion chromatogram of the last sample analysed on the GC-MS.

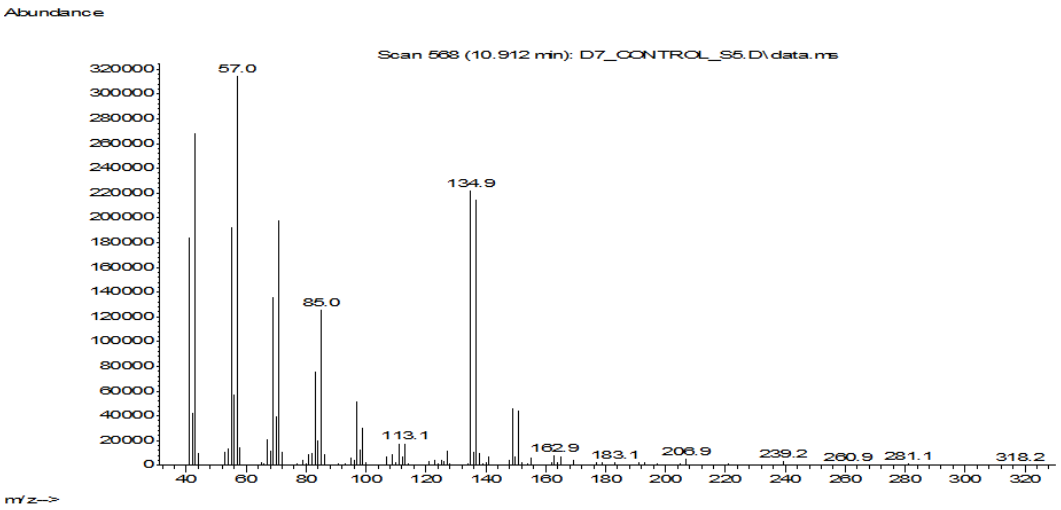


Figure 175: Mass spectrum of Bromoheptadecane within the last analysed sample, with retention time shown.

Hydrocarbon peaks were identified as shown in chapter 2, using the pattern produced by the detection of fragment ions at different abundancies. The molecular ion and the high intensity fragment ions showing where a hydrocarbon chain has cleaved therefore were indicating the methyl group position. Peaks were integrated using Chemstation software where parameters were chosen to give hydrocarbons with a chain length between 18 and 35 and above 0.5% abundance. The labelled chromatogram in Figure 176 shows the position of identified hydrocarbons.

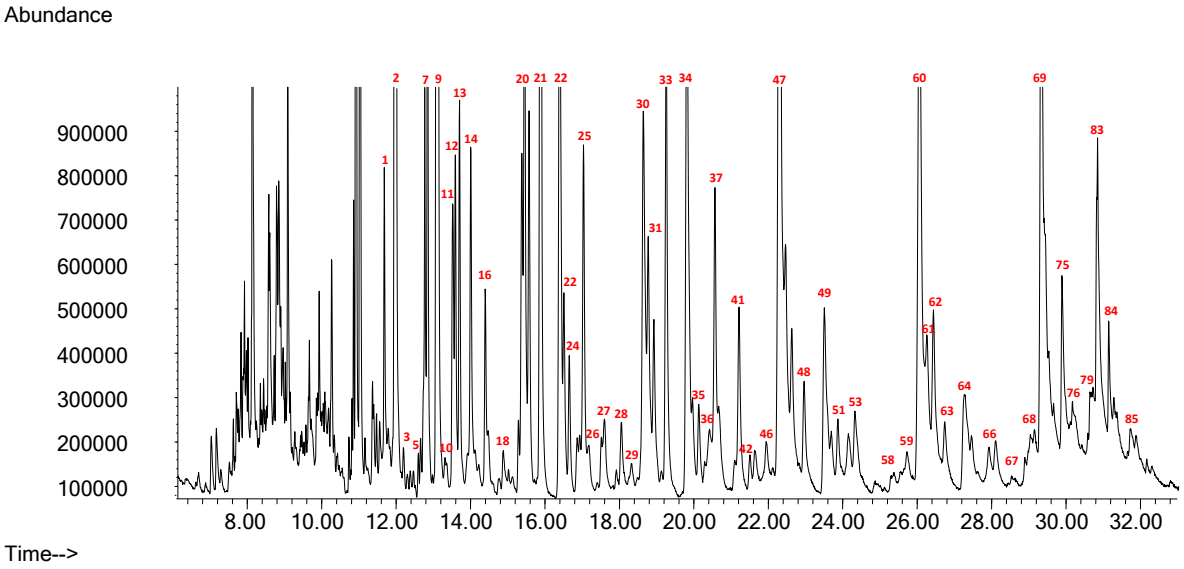


Figure 176: Chromatogram with hydrocarbon peaks labelled.

Principle component analysis was carried out using the integrated hydrocarbon peaks and their corresponding peak areas. All hydrocarbons observed are included in the analysis. Each sample containing all hydrocarbons observed within that treatment on that sampling day was treated as an individual sample and then grouped after analysis according to sampling day. An overview of all sampling days is shown in Figure 177. All hydrocarbon samples are plotted but some samples appear on top of one another. Days 1 and 2 are shown to cluster; this had been seen in analysis previously concerning first instar larvae. Day 3 clusters above this slightly more elongated, day 4 to the right of this. Day 5 appears on the right-hand side of the plot, slightly more spread out than the other sampling days. Day 6 then clusters along the bottom of the plot. Days 7 and 8 appear together close to the left-hand side of the elongated day 6 sample cluster. Days 9, 10, and 11 contain only the control and paracetamol, this does not allow sufficient data to observe and interpret the pattern in these developmental days. As has been shown in

previous research [234], a systematic clockwise pattern is observed when considering all sampling days together.

Each developmental day will be considered separately, hydrocarbons identified and listed alongside the peak area, the control chromatogram, and the data from one population feeding on a drug diet is also shown for comparison. Peak number is also listed and refers to Figure 176. Hydrocarbons present above 0.5% appeared to mainly consist of branched mono-methyl alkanes across all development days. Other hydrocarbons may be present in smaller concentrations; it has been suggested in previous analysis that separation and subsequent analysis of branched alkenes provides the best method for clear separation [221].

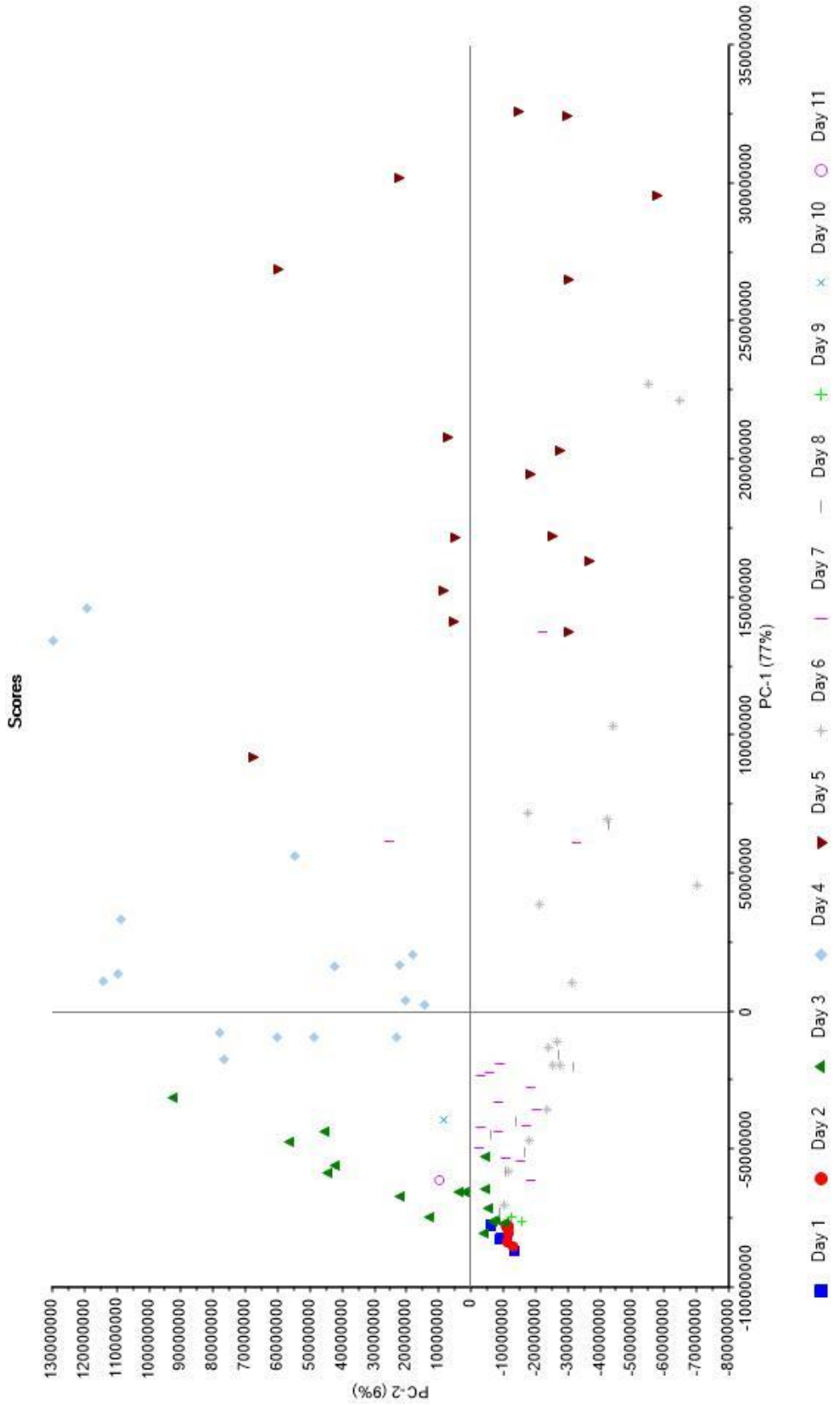


Figure 177: PCA plot of all development days analysed using identified hydrocarbons and peak areas.

6.3.4 Day 1

Chromatogram profiles of day 1 samples contained 27 compounds identified as hydrocarbons (Table 32). Hydrocarbons were observed with a chain length that ranged between 21 and 33. 66.7% of hydrocarbons identified at this sampling point were shown to be branched mono-methyl alkanes (Table 34); the profile across all sampling days appears to be dominated with mono-methyl alkanes. Distinction cannot be made in relation to chemical changes in the hydrocarbon profile at this stage. It is unlikely that any differences would be observed at this point due to drug presence given the short amount of time spent on the diet. A chromatogram is shown in Figure 178 for comparison purposes.

Hydrocarbon Identification	Peak Number	Rentention Time	Control	MDA	Ivory Wave	5EAPB	Benzocaine	AMT	Pink Panther	6+5 APB	6APB	Benzofury Green	Benzofury Beige	Benzofury Blue	Synthacaine	Caffiene	Blow	Paracetamol
9+11-Methylhenicosane	1	12.152	2.41	1.90	0.00	2.92	2.07	0.00	2.77	2.48	2.67	2.56	0.00	0.00	2.89	2.64	0.00	1.98
7-Methylhenicosane	5	12.56	0.91	0.00	2.58	1.10	1.25	1.04	1.04	0.93	1.00	0.96	2.05	2.58	1.08	0.99	2.58	0.00
6-Methylhenicosane	6	12.662	2.88	3.94	6.40	3.49	1.25	3.31	0.00	0.00	3.19	3.06	5.07	6.40	0.00	0.00	6.40	4.10
Docosene	8	12.815	3.72	5.84	9.93	3.27	4.88	4.28	0.00	3.83	4.13	3.96	7.88	9.93	0.00	4.08	9.93	6.07
Docosane	9	13.087	3.63	8.80	6.40	4.28	4.02	4.17	4.17	3.73	4.02	3.85	5.08	6.40	4.34	3.97	6.40	9.15
11-Methyldocosane	10	13.35	2.18	1.29	0.00	2.64	1.65	2.50	2.51	2.24	2.42	2.32	0.00	0.00	2.61	2.39	0.00	1.34
10-Methyldocosane	11	13.58	1.92	2.42	0.00	2.33	2.65	2.21	2.21	1.98	2.13	2.04	0.00	0.00	2.30	2.11	0.00	0.00
9-Methyldocosane	12	13.699	0.88	1.45	2.51	1.06	0.00	1.01	1.01	0.90	0.97	0.93	1.99	2.51	1.05	0.00	2.51	0.00
7-Methyldocosane	13	13.894	4.48	1.34	0.00	5.42	2.69	5.14	5.15	4.61	4.96	4.76	4.43	0.00	5.36	4.90	0.00	1.40
6-Methyldocosane	14	13.996	2.63	2.32	6.67	3.19	2.02	3.03	3.03	2.71	0.00	0.00	5.29	6.67	3.15	2.89	6.67	2.41
5-Methyldocosane	15	14.208	2.07	1.29	0.00	2.50	2.84	2.38	2.38	2.13	2.29	0.00	0.00	0.00	2.48	2.27	0.00	1.35
Tricosene	18	15.559	4.51	4.19	0.00	5.46	6.21	5.18	5.19	4.65	5.00	4.80	0.00	0.00	5.40	4.94	0.00	4.36
Tricosene	19	15.721	2.10	1.95	6.00	2.55	2.89	2.41	2.42	2.16	2.33	2.23	4.76	6.00	2.52	2.30	6.00	2.03
Tricosane	20	15.857	8.96	8.32	0.00	10.85	12.33	10.29	10.31	9.23	9.93	9.52	0.00	0.00	10.73	9.82	0.00	8.66
9+11-Methyltricosane	21	16.383	1.73	2.44	5.07	2.09	2.38	0.00	1.99	1.78	1.92	1.84	4.02	5.07	2.07	1.90	5.07	2.53
7-Methyltricosane	22	16.485	8.80	9.62	10.35	3.32	5.29	0.00	10.13	9.06	9.76	9.36	8.21	10.35	10.54	9.64	10.35	10.01
Tetracosane	25	17.029	3.31	0.00	0.00	4.01	4.56	3.81	3.81	3.41	3.67	3.52	7.50	0.00	3.97	3.63	0.00	0.00
Tetracosane	26	17.496	3.87	3.59	0.00	4.68	5.32	4.44	0.00	3.98	4.29	4.11	8.76	0.00	0.00	4.24	0.00	3.74
10+8-Methyltetracosane	27	17.581	7.62	0.00	8.94	3.22	3.45	8.75	8.77	7.85	8.45	8.10	7.09	8.94	9.12	8.35	8.94	0.00
Pentacosene	32	18.957	2.18	2.03	6.24	2.64	0.00	2.51	2.51	2.25	0.00	2.32	4.95	6.24	2.62	2.39	6.24	2.11
Pentacosane	33	19.246	4.97	8.99	5.70	6.02	6.84	5.71	5.72	5.12	0.00	5.28	4.52	5.70	5.95	0.00	5.70	9.35
9+11-Methylpentacosane	34	19.815	3.39	3.14	0.00	4.10	4.66	3.89	3.90	3.49	3.75	3.60	0.00	0.00	0.00	3.71	0.00	3.27
Mono-methyl branched hexacosane	41	21.073	2.66	2.47	0.00	0.00	3.67	3.06	3.07	2.74	2.95	2.83	0.00	0.00	3.19	2.92	0.00	2.57
4-Methylhexacosane	42	21.192	10.14	9.42	10.15	9.12	6.02	11.65	11.67	10.44	11.24	10.78	8.05	10.15	12.14	11.11	10.15	9.80
Nonacosane	48	22.942	2.76	8.33	7.86	3.34	3.79	3.16	3.17	2.84	3.05	2.93	6.24	7.86	3.30	3.02	7.86	8.67
11+13-Methylnonacosane	49	23.511	2.68	2.49	0.00	3.24	3.68	3.08	3.08	2.76	2.97	2.85	0.00	0.00	3.20	2.93	0.00	2.59
5-Methylhentriacontane	67	28.651	1.22	1.13	3.48	1.47	1.68	1.40	0.00	1.25	1.35	0.00	2.76	3.48	0.00	1.33	3.48	1.18
Tritriacontene	72	29.627	0.79	0.73	0.00	0.95	1.08	0.90	0.00	0.81	0.87	0.83	0.00	0.00	0.00	0.86	0.00	0.76
4-Methyltritriacontane	77	30.477	0.60	0.56	1.72	0.73	0.83	0.69	0.00	0.62	0.67	0.64	1.37	1.72	0.00	0.66	1.72	0.58

Table 33: List of hydrocarbons identified at sampling day 1 (%).

Table 34: A breakdown of the hydrocarbon types analysed at sampling day 1.

Day 1	Percentage
Alkenes	14.8
Alkanes	18.5
Mono-methyl branched Alkanes	66.7

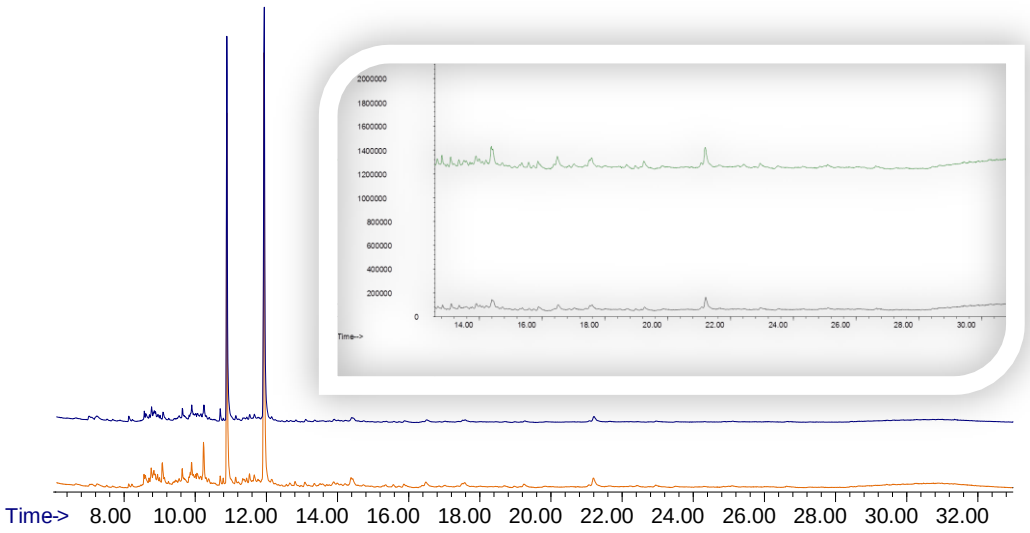


Figure 178: Day 1 control and Synthacaine diet chromatograms for comparison.

6.3.5 Day 2

Chromatogram profiles for day 2 of sampling identified 18 hydrocarbons (Table 35). Hydrocarbons identified had a chain length between 21 and 26 and 66.7% of identified components were shown to be mono-methyl alkanes (Table 36).No obvious changes in the chemical composition are observed at this sampling point and the treatments appeared to cluster together revealing no big differences.

Hydrocarbon Identification	Peak Number	Retention Time	Control	MDA	Ivory Wave	5EAPB	Benzocaine	AMT	Pink Panther	6+5APB	6APB	Benzofury Green	Benzofury Beige	Benzofury Blue	Synthacaine	Caffiene	Blow	Panacetamol
6-Methyheneicosane	6	12.662	0.00	2.58	0.00	1.51	1.60	0.00	1.83	1.26	2.58	1.46	0.00	1.09	1.83	1.34	0.00	0.00
Docosene	8	12.815	12.50	15.81	2.75	9.24	10.11	2.75	11.20	7.96	15.81	16.05	2.91	11.94	11.20	14.75	2.91	13.40
Docosane	9	13.087	12.18	15.43	5.01	9.01	11.01	5.01	10.93	8.67	15.43	14.27	5.29	10.62	10.93	13.11	5.29	13.06
10-Methyldocosane	11	13.58	6.74	8.64	0.00	5.05	6.57	0.00	6.12	5.17	8.64	8.44	0.00	6.28	6.12	7.75	0.00	0.00
9-Methyldocosane	12	13.699	9.50	5.13	10.59	2.99	4.87	10.59	3.63	3.83	5.13	4.49	11.19	3.34	3.63	4.13	11.19	10.19
7-Methyldocosane	13	13.894	0.00	0.00	3.64	0.00	0.00	3.64	0.00	0.00	0.00	0.00	3.85	0.00	0.00	0.00	3.85	0.00
6-Methyldocosane	14	13.996	7.59	7.03	3.24	4.11	7.81	3.24	7.86	6.15	7.03	7.15	3.42	5.32	7.86	6.57	3.42	8.14
Tricosane	20	15.857	9.22	9.88	5.35	5.77	8.80	5.35	9.54	6.93	9.88	10.37	0.00	9.32	9.54	9.53	0.00	9.89
9+11-Methyltricosane	21	16.383	4.11	5.55	0.00	3.24	4.17	0.00	4.15	3.29	5.55	6.11	0.00	4.42	4.15	5.62	0.00	4.41
7-Methyltricosane	22	16.485	8.09	6.71	12.38	8.46	10.32	12.38	10.25	8.12	6.71	6.81	13.08	10.92	10.25	6.25	13.08	8.67
Tetracosane	25	17.089	4.82	3.81	0.00	3.15	5.06	0.00	5.63	3.09	3.81	5.46	0.00	5.56	5.03	5.02	0.00	5.17
Tetracosane	26	17.406	1.35	1.81	0.00	0.97	1.18	0.00	1.17	0.93	1.81	0.00	0.00	1.25	1.17	0.00	0.00	1.45
10+8-Methylteracosane	27	17.581	1.27	0.00	0.00	0.00	1.09	0.00	1.08	0.00	0.00	0.00	0.00	1.15	1.08	0.00	0.00	1.37
Pentacosene	32	18.957	2.54	3.36	0.00	0.00	4.10	0.00	4.07	0.00	3.36	3.32	0.00	4.34	4.07	3.05	0.00	2.72
Pentacosane	33	19.246	2.92	4.04	1.80	1.44	2.81	1.80	2.79	1.38	4.04	2.71	1.91	2.97	2.79	2.49	1.91	3.13
9+11-Methylpentacosane	34	19.815	0.00	0.00	0.00	0.00	2.43	0.00	2.42	0.00	0.00	0.00	0.00	2.58	2.42	0.00	0.00	0.00
5-Methylpentacosane	36	20.147	0.00	0.00	5.20	4.14	0.00	5.20	0.00	3.98	0.00	0.00	5.49	0.00	0.00	6.61	5.49	0.00
Mono-methyl branched hexacosane	41	21.073	0.00	0.00	1.16	0.93	0.00	1.16	0.00	0.89	0.00	0.00	1.23	0.00	0.00	1.48	1.23	0.00
4-Methylhexacosane	42	21.192	17.16	10.21	48.88	38.99	18.06	48.88	17.94	37.45	10.21	13.36	51.65	19.11	17.94	12.28	51.65	18.40

Table 35: List of all hydrocarbons identified at sampling day 2 (%).

Table 36: A breakdown of the hydrocarbon types analysed at sampling day 2.

Day 2	Percentage
Alkenes	11.1
Alkanes	22.2
Mono-methyl branched Alkanes	66.7

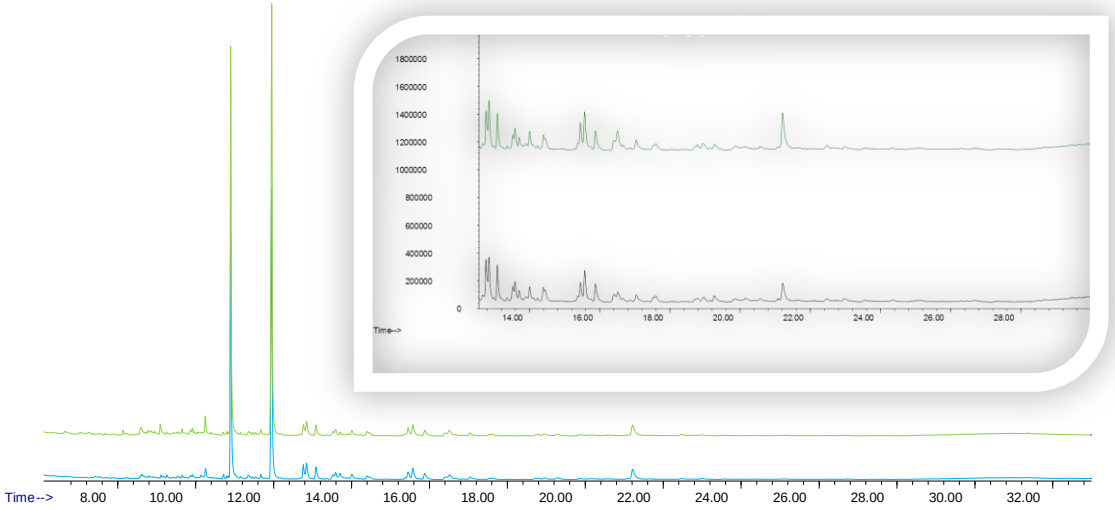


Figure 179: Day 2 control and Benzofury Beige diet chromatograms for comparison.

Principle component analysis of day 1 and 2

Principle component analysis of sampling days 1 and 2 showed clear separation, 81% of data variance was displayed with PC1 and 2. Previously when analysing the whole profile, days 1 and 2 were shown to cluster. Separate analysis shows tight clustering for day 1 samples; day 2 samples are clearly distinguishable but are spread across three quadrants of the plot.

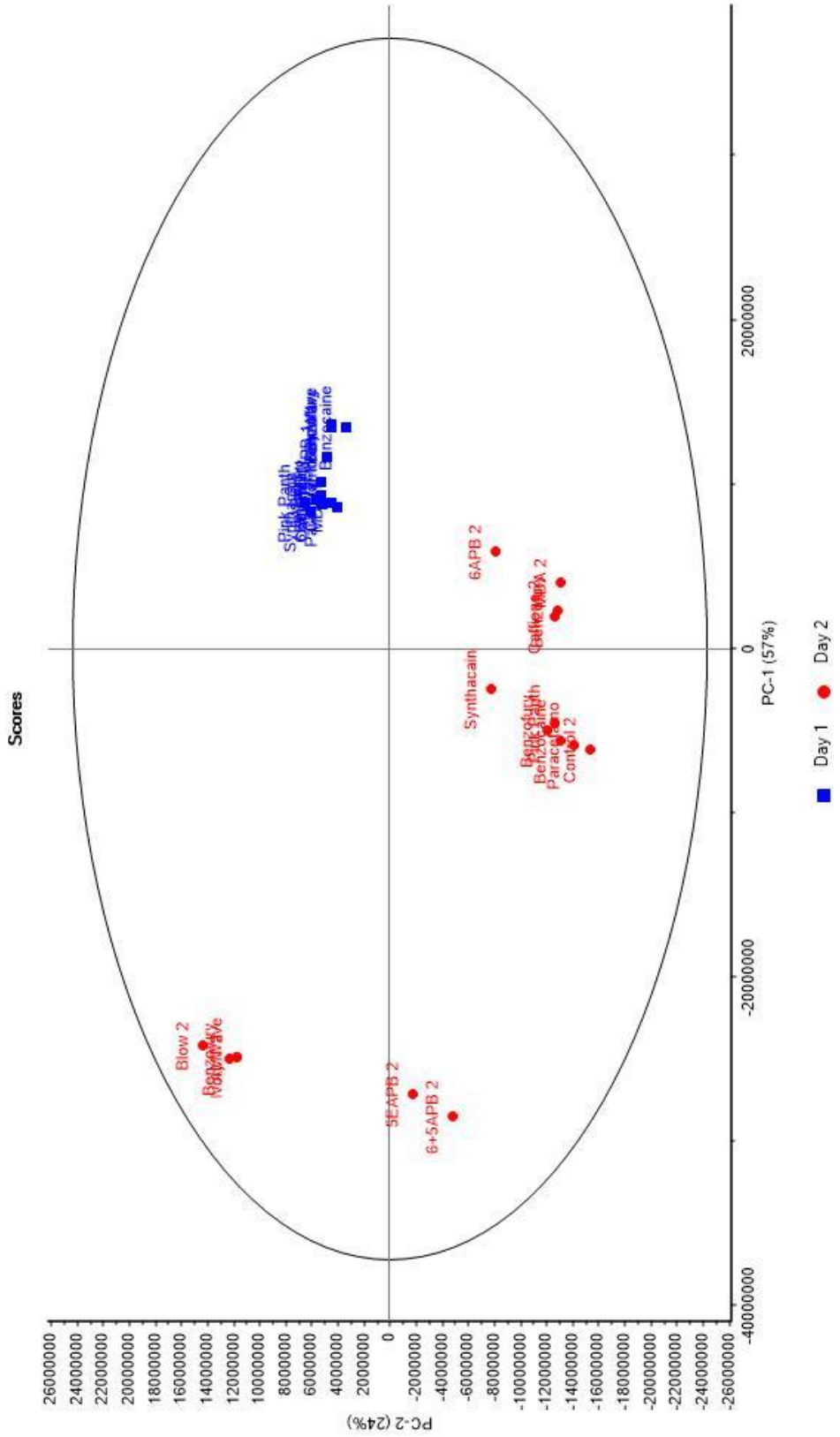


Figure 180: PCA plot of development days 1 and 2 analysed using identified hydrocarbons and peak areas.

6.3.6 Day 3

On sampling day 3, 48 individual hydrocarbons were detected with a chain length between 21 and 33 (Table 37). 72.9% of hydrocarbons analysed were mono-methyl alkanes (Table 38). The majority of samples were very similar to the control sample. The sample from the AMT population appears to contain fewer hydrocarbons than other populations, with a total of 18, it is possible that this depletion of hydrocarbons is due to low concentration or sample variability, the sample was monitored throughout the next development days to determine if a similar trend continued. No further chemical changes were obvious.

Table 37: List of all hydrocarbons identified at sampling day 3 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	MDA	IvoryWave	5EAPB	Benzocaine	AMT	Pink Panther	6+5APB	6APB	Benzofury Green	Benzofury Beige	Benzofury Blue	Synthacaine	Caffiene	Blow	Paracetamol
9+11-Methylhenicosane	1	12.152	0.00	0.00	0.00	0.34	1.12	0.00	0.00	0.00	0.71	0.00	0.00	0.00	0.00	0.80	0.00	0.55
Mono-methyl branched henicosane	3	12.339	0.38	0.95	0.00	0.00	0.56	0.00	0.00	0.61	0.76	0.00	0.49	0.00	0.00	0.00	0.00	0.63
6-Methylhenicosane	6	12.662	0.47	1.19	1.72	1.72	0.64	11.66	0.39	3.01	0.78	1.18	0.77	0.00	0.48	0.77	0.96	1.53
Docosene	8	12.815	1.65	4.16	5.23	1.59	1.08	13.50	1.60	3.36	3.09	4.71	2.10	1.22	2.06	9.99	10.43	0.87
Docosane	9	13.087	4.75	11.98	6.14	3.57	2.75	9.73	3.45	3.75	8.62	10.19	6.32	3.18	2.60	7.01	10.49	14.13
11-Methyldocosane	10	13.35	0.21	0.54	1.02	0.36	0.97	0.70	0.00	0.36	1.77	0.62	0.49	0.00	0.31	0.75	0.00	0.68
10-Methyldocosane	11	13.58	1.33	3.34	3.19	1.09	1.76	6.19	0.91	0.00	4.44	2.50	2.09	0.91	1.40	4.90	5.55	3.07
9-Methyldocosane	12	13.699	1.16	2.92	1.73	1.69	2.26	3.83	0.70	0.73	2.87	4.26	2.07	1.10	1.53	6.61	3.36	1.90
7-Methyldocosane	13	13.894	0.54	1.36	3.45	0.93	2.53	1.56	1.11	0.64	3.74	2.12	1.25	0.00	0.86	0.00	2.70	1.03
6-Methyldocosane	14	13.996	1.63	4.12	3.44	1.46	2.46	4.64	0.00	1.49	3.78	2.40	2.32	1.40	1.52	4.01	6.20	2.60
5-Methyldocosane	15	14.208	0.00	0.00	0.00	0.74	3.25	0.00	0.00	0.00	1.61	2.78	0.59	0.54	0.00	0.00	0.00	0.77
2-Methyldocosane	17	14.905	1.18	2.99	0.00	1.13	1.29	0.00	0.00	0.00	0.00	0.00	2.41	0.93	0.00	0.00	0.00	0.00
Tricosene	18	15.559	4.23	10.67	6.73	2.90	4.42	12.08	3.94	10.98	2.86	5.65	3.14	4.75	5.37	10.61	11.31	2.76
Tricosene	19	15.721	0.00	0.95	0.00	0.00	4.23	7.26	0.00	0.00	1.10	0.00	7.37	0.00	0.00	0.00	0.00	0.92
Tricosane	20	15.857	6.79	6.34	6.63	4.67	0.00	0.00	5.55	5.02	7.38	9.94	0.00	5.31	3.72	6.81	8.67	9.22
9+11-Methyltricosane	21	16.383	2.26	3.54	3.14	1.59	1.95	3.46	1.31	1.92	4.12	3.44	2.86	1.73	1.79	3.19	3.53	5.41
7-Methyltricosane	22	16.485	2.52	6.02	4.23	2.12	2.38	5.30	1.30	2.58	7.01	4.19	4.06	2.07	2.03	6.87	4.89	3.92
6-Methyltricosane	23	16.63	1.10	0.00	0.00	0.65	1.08	0.00	0.63	0.97	0.00	0.00	1.45	1.00	0.91	0.00	0.00	1.39
Mono-methyl branched tricosane	24	16.85	0.50	1.59	0.00	0.00	1.00	0.00	0.29	0.00	1.86	0.00	0.74	0.00	0.43	0.00	0.00	1.58
Tetracosane	25	17.029	3.46	3.63	4.22	2.23	4.10	3.64	2.23	3.05	4.23	3.00	3.68	3.39	2.55	3.25	3.82	2.74
Tetracosane	26	17.496	0.81	2.53	2.19	0.67	1.71	1.11	0.84	0.96	2.94	1.49	1.27	0.37	0.68	1.47	1.25	1.84
Mono-methyl branched tetracosane	28	18.159	3.21	0.00	0.00	1.41	0.00	0.00	0.00	0.00	0.00	0.00	4.51	1.54	0.00	0.00	0.00	1.20
Mono-methyl branched tetracosane	29	18.277	3.55	0.00	1.70	0.61	0.00	0.00	0.00	0.00	0.00	7.01	0.00	0.30	0.00	1.30	1.28	1.95
Pentacosene	32	18.957	0.00	1.15	4.08	3.12	5.28	2.16	4.33	6.13	1.34	2.27	3.42	6.47	5.47	2.50	2.25	1.35
Pentacosane	33	19.246	7.57	4.15	5.28	5.71	3.94	3.03	7.51	5.32	4.84	6.67	6.38	6.91	4.57	2.41	3.00	6.35
9+11-Methylpentacosane	34	19.815	2.55	3.53	0.00	1.28	2.07	2.24	0.69	1.73	4.11	1.28	3.27	2.22	1.79	1.72	0.00	6.22
9-Methylpentacosane	35	19.943	1.41	0.00	3.30	1.39	1.09	0.00	0.85	0.84	0.00	1.18	2.08	1.62	1.05	0.00	0.00	0.00
5-Methylpentacosane	36	20.147	1.20	2.98	0.00	1.47	0.76	0.00	0.55	2.33	3.47	0.00	1.69	1.34	0.79	0.00	1.40	1.12
Hexacosane	39	20.546	5.79	0.00	3.08	5.43	3.10	0.00	4.65	3.65	0.00	1.80	5.89	6.53	3.50	0.00	0.00	2.10
Mono-methyl branched hexacosane	41	21.073	0.68	1.50	0.00	0.73	0.71	0.00	0.93	0.61	1.75	0.94	0.93	0.41	0.65	0.86	0.00	1.09
4-Methylhexacosane	42	21.192	2.57	8.91	5.74	3.61	3.30	4.74	0.71	2.97	10.38	5.62	5.78	3.33	3.64	23.10	16.56	2.63
Nonacosene	47	22.44	3.72	1.25	4.23	4.20	4.20	1.27	4.80	5.26	1.46	2.67	2.83	5.25	4.29	1.07	2.35	1.83
Nonacosane	48	22.942	7.27	2.90	3.61	7.46	4.37	1.91	8.59	5.94	3.38	5.54	3.88	7.57	5.94	0.00	0.00	4.82
11+13-Methylnonacosane	49	23.511	1.44	1.31	2.39	0.83	0.42	0.00	0.88	0.76	1.53	1.05	0.95	0.80	0.88	0.00	0.00	2.89
7+9-Methylnonacosane	50	23.553	0.00	0.00	0.00	0.85	0.30	0.00	0.75	0.50	0.00	0.00	0.62	0.73	0.67	0.00	0.00	0.00
7-Methylnonacosane	51	23.672	2.05	0.00	2.99	2.96	1.96	0.00	2.58	1.84	0.00	0.00	2.62	2.65	2.61	0.00	0.00	0.54
5-Methylnonacosane	52	23.842	1.62	0.00	0.00	2.13	1.34	0.00	2.13	1.27	0.00	0.00	1.93	2.32	2.17	0.00	0.00	0.00
Mono-methyl branched nonacosane	53	24.309	2.36	0.00	1.98	4.14	2.15	0.00	3.90	2.19	0.00	1.34	2.74	4.09	3.96	0.00	0.00	0.28
4-Methylnonacosane	54	24.496	0.00	0.00	0.00	0.00	0.00	0.00	0.70	0.00	0.00	0.00	0.00	1.07	0.68	0.00	0.00	0.00
Mono-methyl branched nonacosane	55	24.989	0.00	0.55	0.00	0.00	0.00	0.00	0.61	0.00	0.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mono-methyl branched nonacosane	56	25.108	0.00	0.35	0.00	0.00	0.00	0.00	0.72	0.00	0.41	0.00	0.00	0.00	0.85	0.00	0.00	0.00
Hentriacontene	60	26.017	0.34	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.87
Hentriacontene	61	26.246	3.30	0.00	2.73	4.07	4.94	0.00	6.08	4.66	0.00	1.19	1.40	3.18	4.99	0.00	0.00	0.00
Hentriacontene	62	26.416	9.49	0.00	5.33	10.52	12.48	0.00	16.60	12.90	0.00	2.12	1.41	10.66	15.36	0.00	0.00	0.00
Hentriacontane	63	26.722	0.00	0.27	0.00	3.24	1.16	0.00	0.00	0.00	0.31	0.00	0.00	0.00	1.30	0.00	0.00	0.00
9+11-Methylhentriacontane	64	27.283	0.47	0.00	0.00	0.30	0.00	0.00	0.93	0.00	0.00	0.00	0.00	0.00	0.68	0.00	0.00	0.21
8-Methylhentriacontane	65	27.351	0.41	0.00	0.00	0.39	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.75	0.00	0.00	0.00	0.00
6-Methylhentriacontane	66	27.444	1.02	0.00	0.00	0.82	0.60	0.00	0.93	0.00	0.00	0.00	0.06	0.75	0.54	0.00	0.00	0.00
5-Methylhentriacontane	67	28.651	0.00	0.52	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.00	0.28	0.00	0.00	0.00	0.00	0.39
Tritriacontene	69	29.305	0.26	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.00	1.51
Tritriacontene	70	29.424	1.02	0.74	0.51	1.48	2.05	0.00	1.79	1.05	0.86	0.54	0.73	0.69	1.83	0.00	0.00	1.21
Tritriacontene	71	29.509	0.97	0.00	0.00	1.03	2.24	0.00	1.94	0.65	0.00	0.00	0.21	0.89	2.61	0.00	0.00	0.00
Tritriacontene	72	29.627	0.53	0.46	0.00	0.76	0.00	0.00	0.55	0.00	0.53	0.00	0.28	0.00	0.00	0.00	0.00	0.87
7-Methyltritriacontane	74	29.891	0.21	0.40	0.00	0.65	0.00	0.00	0.40	0.00	0.47	0.00	0.39	0.00	0.93	0.00	0.00	1.56
4-Methyltritriacontane	77	30.477	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.51

Table 38: A breakdown of the hydrocarbon types analysed at sampling day 3.

Day 3	Percentage
Alkenes	12.5
Alkanes	14.6
Mono-methyl branched Alkanes	72.9

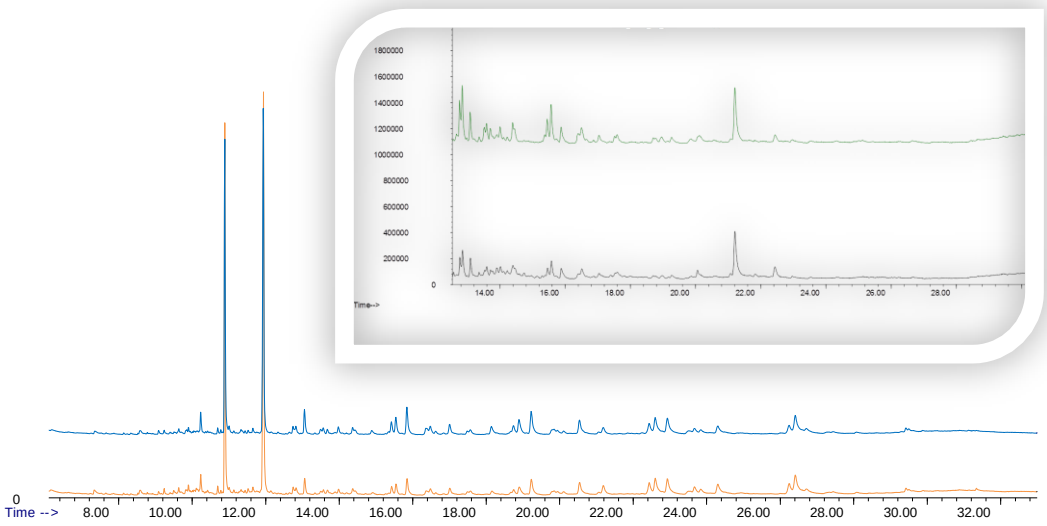


Figure 181: Day 3 control and Blow diet chromatograms for comparison.

6.3.7 Day 4

On Sampling day 4, 52 hydrocarbon peaks were detected (Table 39). 71.2% of these were mono-methyl alkanes (Table 40). Hydrocarbons identified ranged between 21 and 35 chain lengths. No obvious changes were observed when comparing the compounds present across the different diets (Figure 182).

Table 39: List of all hydrocarbons identified at sampling day 4 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	5EAPB	6+5APB	6APB	AMT	Benzofury Beige	Benzofury Blue	Benzocaine	Benzofury Green	Blow	Caffiene	Ivory Wave	Paracetamol	Pink Panther	Synthacaine	MDA
9+11-Methylhenicosane	1	12.152	0.00	0.22	0.00	0.00	0.00	0.38	0.26	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.22	0.00
9-Methylhenicosane	2	12.238	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00
6-Methylhenicosane	6	12.662	0.00	0.35	0.58	0.59	0.00	0.19	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.17	0.57
Docosene	7	12.73	0.24	1.45	3.48	1.31	0.38	0.21	0.44	0.58	0.66	0.44	0.00	0.34	0.00	0.36	0.45	1.25
Docosene	8	12.815	1.17	1.52	2.29	1.90	1.87	1.89	1.96	1.64	1.77	1.84	0.95	1.58	0.73	1.30	1.77	1.82
Docosane	9	13.087	17.42	20.47	7.72	16.82	5.38	12.57	17.49	9.73	15.78	5.30	8.56	7.14	6.09	13.23	15.03	16.08
11-Methyldocosane	10	13.35	0.00	0.18	0.00	0.22	0.15	0.33	0.28	0.18	0.24	0.14	0.00	0.00	0.00	0.11	0.17	0.21
10-Methyldocosane	11	13.58	2.94	1.00	1.07	1.40	0.78	1.92	1.20	1.06	0.89	0.82	0.42	0.74	0.34	0.69	0.92	1.34
9-Methyldocosane	12	13.699	1.86	0.80	0.94	1.90	0.75	1.62	1.03	0.94	0.70	0.77	0.36	0.62	0.31	0.61	0.71	1.81
7-Methyldocosane	13	13.894	0.00	0.48	0.00	0.00	0.35	0.72	1.35	0.00	0.46	0.38	0.00	0.26	0.30	0.22	0.31	0.00
6-Methyldocosane	14	13.996	1.52	1.40	1.26	1.34	1.03	1.44	0.00	1.27	0.91	1.01	0.00	0.88	0.56	0.85	0.92	1.28
5-Methyldocosane	15	14.208	0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00
2-Methyldocosane	17	14.905	0.48	0.00	0.00	0.00	0.00	0.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00
Tricosene	18	15.559	2.00	1.89	3.71	2.48	3.38	2.72	2.40	2.61	2.40	2.92	2.05	2.67	1.49	1.89	2.65	2.37
Tricosane	20	15.857	10.86	11.85	9.46	12.28	7.25	9.28	13.27	11.67	14.39	6.60	10.71	9.80	9.49	13.89	14.94	11.74
9+11-Methyltricosane	21	16.383	2.58	2.31	1.84	2.69	1.13	2.44	2.22	2.17	1.83	1.09	0.79	1.42	0.55	1.39	1.79	2.57
7-Methyltricosane	22	16.485	1.71	1.39	1.25	1.73	1.63	1.35	1.20	1.39	0.90	1.22	0.70	1.04	1.07	0.74	0.97	1.65
6-Methyltricosane	23	16.63	0.79	0.78	0.62	0.90	0.65	0.69	0.67	0.73	0.40	0.51	0.00	0.54	0.38	0.43	0.47	0.86
Mono-methyl branched tricosane	24	16.85	0.00	0.00	0.42	0.61	0.36	0.54	0.00	0.39	0.29	0.00	0.00	0.35	0.00	0.21	0.38	0.58
Tetracosane	25	17.029	0.00	2.11	2.12	2.22	1.89	0.00	0.00	0.00	1.45	1.97	1.50	1.84	1.26	1.64	1.99	2.19
Tetracosane	26	17.496	0.57	0.84	0.56	0.96	0.55	0.76	1.02	0.73	0.73	0.56	0.50	0.58	0.63	0.64	0.80	0.87
10+8-Methyltetracosane	27	17.581	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.00
Mono-methyl branched tetracosane	28	18.159	0.88	0.34	0.00	0.00	0.00	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.35
Mono-methyl branched tetracosane	29	18.277	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.00	0.17	0.31	0.00
Pentacosene	30	18.736	1.57	1.57	2.79	1.41	2.95	2.60	1.29	2.18	1.56	2.95	2.03	2.26	1.72	1.42	1.86	1.63
Pentacosene	31	18.898	1.45	1.36	2.71	1.34	2.61	2.40	1.33	1.80	1.36	2.61	1.96	2.28	1.40	1.37	1.54	1.41
Pentacosane	33	19.246	5.63	6.99	7.63	6.13	6.81	6.13	7.85	7.21	8.42	6.62	8.86	8.15	10.05	9.03	9.36	7.25
9+11-Methylpentacosane	34	19.815	2.31	2.72	1.10	2.46	0.56	2.44	1.93	2.18	1.68	1.09	0.46	0.89	0.49	1.57	1.28	2.82
9-Methylpentacosane	35	19.943	0.64	0.61	0.74	0.60	0.61	0.81	0.56	0.68	0.50	0.54	0.00	0.75	0.40	0.51	0.36	0.63
5-Methylpentacosane	36	20.147	0.55	0.74	0.70	0.84	0.55	0.80	0.72	0.50	0.00	0.59	0.00	0.72	0.77	0.54	0.39	0.77
Hexacosane	39	20.546	3.02	2.79	3.82	2.84	3.08	3.19	2.89	3.03	2.19	3.24	3.04	3.56	3.52	2.77	2.04	2.89
Mono-methyl branched hexacosane	41	21.073	0.48	0.49	0.42	0.62	0.51	0.62	0.62	0.48	0.47	0.53	0.51	0.45	1.14	0.40	0.39	0.51
4-Methylhexacosane	42	21.192	2.74	0.84	0.68	1.94	3.35	1.68	2.47	2.24	2.42	3.88	4.13	1.33	4.97	1.00	0.88	0.87
Heptacosene	43	21.48	0.00	0.00	0.37	0.00	0.62	0.00	0.00	0.34	0.26	0.28	0.00	0.33	0.83	0.43	0.34	0.00
10-Methylheptacosane	45	21.956	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.24	0.00
Nonacosene	47	22.44	2.76	2.82	3.24	1.77	3.04	3.11	1.38	3.53	2.34	3.58	3.24	5.02	4.34	2.80	2.40	2.92
Nonacosane	48	22.942	4.96	4.71	6.23	4.35	7.85	4.57	7.11	7.13	6.11	7.84	9.46	7.34	10.82	6.33	6.05	4.88
11+13-Methylnonacosane	49	23.511	1.23	1.54	1.20	1.38	0.54	1.40	1.23	1.08	1.05	0.58	0.75	0.83	0.58	0.72	1.15	1.59
7+9-Methylnonacosane	50	23.553	0.00	0.00	0.00	0.00	0.46	0.00	0.00	0.45	0.00	0.31	0.42	0.46	0.32	0.43	0.00	0.00
7-Methylnonacosane	51	23.672	0.84	0.81	1.66	0.70	1.47	1.03	0.65	1.42	0.69	1.31	1.52	1.77	1.37	1.32	0.78	0.84
5-Methylnonacosane	52	23.842	0.95	0.77	1.59	0.81	1.37	1.16	0.76	1.11	0.58	1.17	1.72	1.56	1.50	1.32	0.78	0.80
Mono-methyl branched nonacosane	53	24.309	1.27	1.21	2.71	1.22	2.58	1.96	1.23	2.45	1.37	2.56	3.02	2.78	2.53	2.10	1.71	1.25
4-Methylnonacosane	54	24.496	0.00	0.00	0.00	0.00	0.82	0.00	0.00	0.00	0.00	0.88	0.00	0.67	0.51	0.00	0.00	0.00
Mono-methyl branched nonacosane	55	24.989	0.00	0.00	0.00	0.00	0.40	0.00	2.90	1.90	0.00	0.00	0.00	0.33	0.25	0.30	0.00	0.00
Mono-methyl branched nonacosane	56	25.108	0.00	0.00	0.35	0.00	0.67	0.00	0.00	0.00	0.00	0.75	0.35	0.21	0.46	0.49	0.23	0.00
Mono-methyl branched triacontane	59	25.719	3.21	0.00	0.00	0.00	0.46	2.69	0.00	0.00	2.59	1.03	1.05	1.50	1.00	1.87	1.83	0.00
Hentriacontene	60	26.017	2.87	3.02	1.31	3.62	1.35	2.91	1.51	3.74	2.29	4.82	4.62	4.11	4.14	2.95	2.12	3.13
Hentriacontene	61	26.246	6.56	2.30	3.59	1.72	3.51	6.16	2.90	6.94	3.92	12.41	12.50	9.43	8.92	6.15	4.17	2.38
Hentriacontene	62	26.416	0.00	3.62	8.40	2.56	9.72	0.00	0.00	0.42	0.00	0.00	0.00	0.00	0.45	0.00	0.13	3.75
Hentriacontane	63	26.722	3.37	3.24	4.61	3.41	5.02	3.65	6.52	5.73	5.80	6.45	7.03	5.54	6.19	5.35	5.38	3.35
6-Methylhentriacontane	66	27.444	0.15	0.69	0.97	0.52	0.53	0.00	0.00	0.38	0.00	0.69	0.62	0.48	0.29	0.35	0.17	0.72
5-Methylhentriacontane	67	28.651	0.00	0.00	0.00	0.00	0.26	0.12	0.00	0.00	0.00	0.24	0.00	0.14	0.11	0.24	0.15	0.00
Tritriacontene	69	29.305	2.58	2.26	1.07	3.44	1.45	2.56	2.66	1.57	2.36	0.81	0.84	1.34	1.11	1.92	2.03	2.34
Tritriacontene	70	29.424	1.81	1.76	1.89	1.57	2.14	1.89	1.63	2.20	1.77	2.36	2.08	2.01	2.20	1.96	1.70	1.82
Tritriacontene	71	29.509	1.46	1.00	1.47	0.84	2.24	1.25	0.89	1.51	1.11	2.35	2.13	1.60	2.05	1.50	1.17	1.04
Tritriacontene	72	29.627	0.68	0.65	0.69	1.20	1.68	0.82	1.78	1.46	1.78	1.24	0.79	1.00	1.69	1.50	1.68	0.67
7-Methyltritriacontane	74	29.891	0.55	0.44	0.37	1.13	0.74	0.58	0.95	0.93	1.55	0.34	0.00	0.48	0.00	1.03	0.99	0.46
6-Methltetratriacontane	80	30.74	0.46	0.00	0.00	0.63	0.72	0.45	0.00	0.00	0.00	0.11	0.00	0.05	0.00	0.23	0.28	0.00
Pentatriacontene	81	30.825	0.70	1.67	0.39	1.60	0.66	1.92	1.13	0.32	1.58	0.14	0.35	0.45	0.41	1.15	0.70	1.73
Mono-methyl branched pentatriacontane	84	31.267	0.18	0.00	0.00	0.00	0.27	0.16	0.31	0.00	0.00	0.13	0.00	0.09	0.27	0.13	0.12	0.00

Table 40: A breakdown of the hydrocarbon types analysed at sampling day 4.

Day 4	Percentage
Alkenes	15.4
Alkanes	13.5
Mono-methyl branched Alkanes	71.2

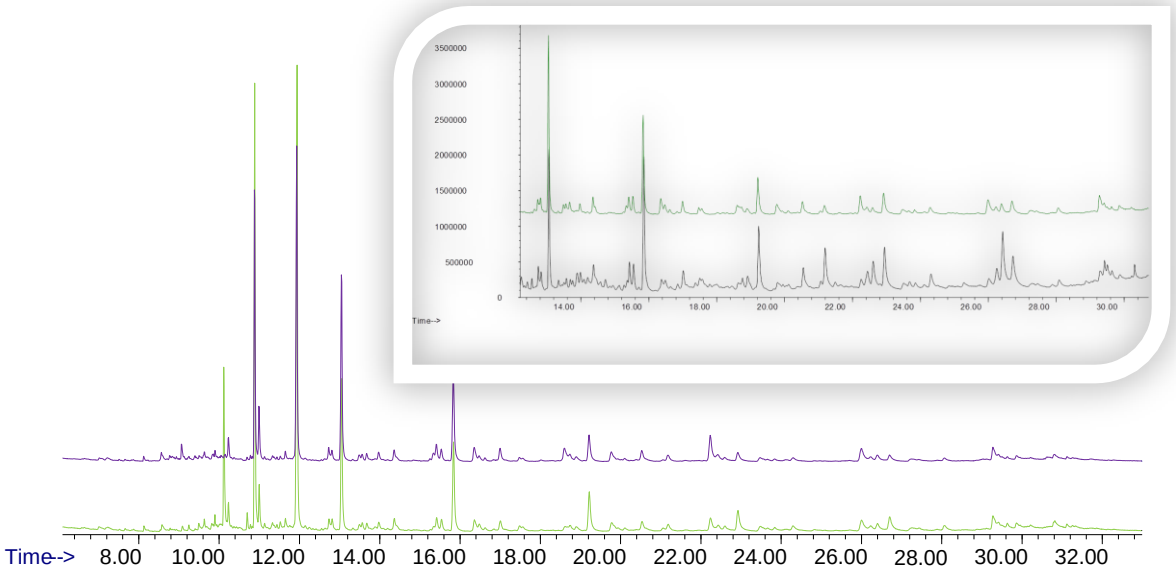


Figure 182: Day 4 control and Caffeine diet chromatograms for comparison.

Principle component analysis of day 3 and 4: Principle component analysis of day 3 and 4 using PC 1 and PC 2 displaying 83% of the data variance showed a clear distinction between sampling days (Figure 183). It is apparent that the ratios of the hydrocarbons present are just as important in defining how similar the cuticular hydrocarbon profile appears. The main bulk of samples for both day 3 and day 4 cluster individually, with the odd sample appearing to move further away. Pink panther is one diet addition, which within these two sampling days appears further away from the main cluster of samples. It is possible that this variation observed is natural; the sample still lies within an area where it would be estimated at the correct age. Previously during analysis all of sampling days together, 3 and 4 were observed to separate.

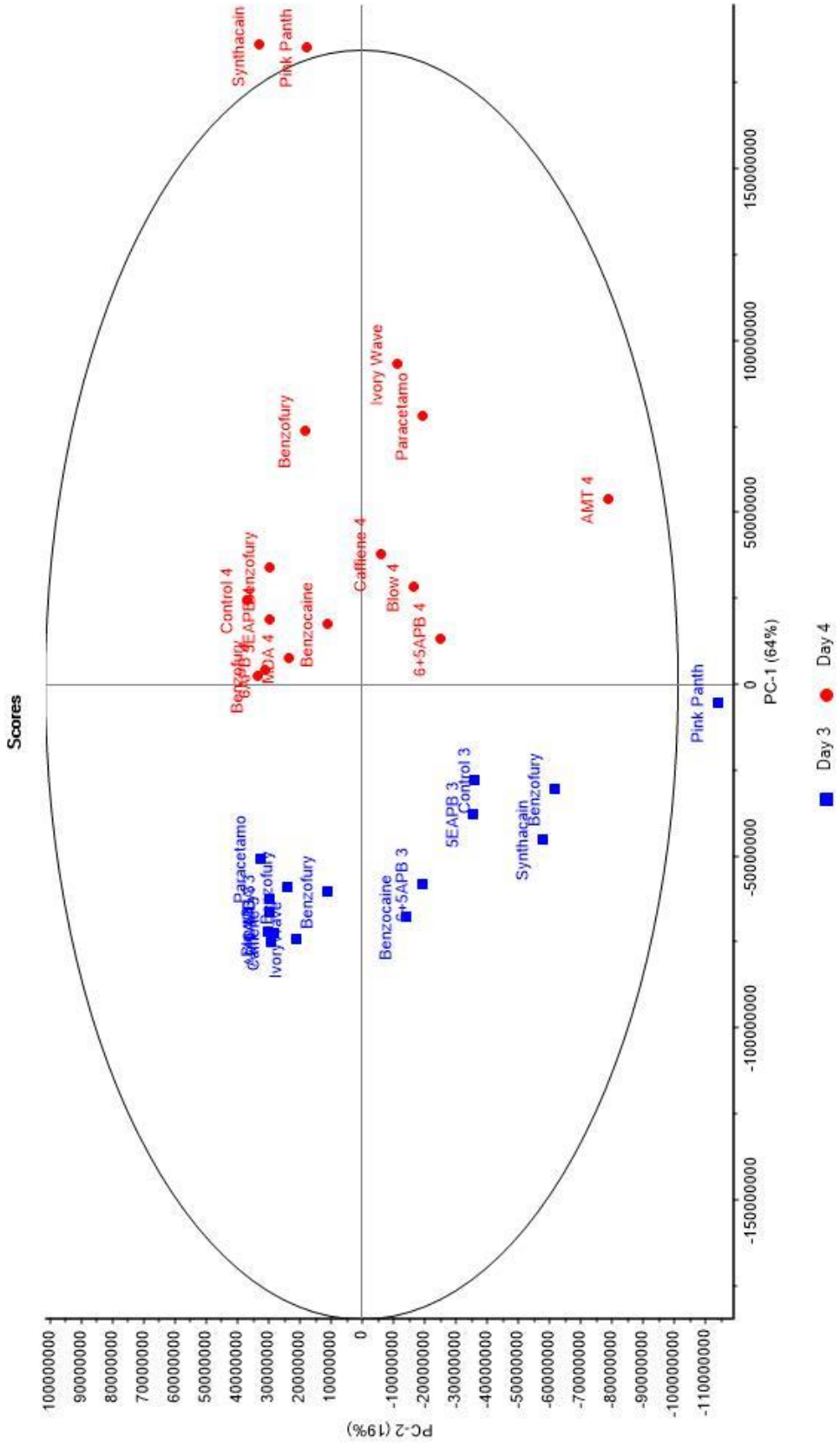


Figure 183: PCA plot of development days 3 and 4 analysed using identified hydrocarbons and peak areas.

6.3.8 Day 5

At day 5, 65 hydrocarbons were observed (Table 41), 75.4% of those were mono-methyl alkanes and ranged between 21 and 35 in chain length (Table 42). When comparing the hydrocarbons identified no obvious differences were observed (Figure 184). Previously at day 3 the AMT population was noted as containing fewer hydrocarbons, this no longer appears to be the case and was therefore likely due to natural sample variance or potentially hydrocarbons were present in lower concentrations due to potential variance in the sampling procedure such as solvent extraction duration.

Table 41: List of all hydrocarbons identified at sampling day 5 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	5EAPB	6+5APB	6APB	AMT	Benzofury Beige	Benzofury Blue	Benzocaine	Benzofury Green	Blow	Caffiene	Ivory Wave	Paracetamol	Pink Panther	Synthacaine	MDA
9+11-Methylhenicosane	1	12.152	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.17	0.00	0.14	0.24	0.16	0.13	0.00
9-Methylhenicosane	2	12.238	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.07	0.00
Mono-methyl branched henicosane	3	12.339	0.15	0.13	0.00	0.23	0.12	0.18	0.18	0.00	0.00	0.00	0.11	0.00	0.09	0.08	0.06	0.20
7-Methylhenicosane	5	12.56	0.00	0.07	0.00	0.00	0.09	0.10	0.09	0.00	0.07	0.11	0.09	0.13	0.10	0.12	0.12	0.00
6-Methylhenicosane	6	12.662	0.17	0.00	0.00	0.42	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.43	0.20	0.00	0.00	0.35
Docosene	7	12.73	1.48	0.81	3.54	0.24	1.33	0.89	1.06	1.84	3.48	2.26	1.64	2.23	2.71	2.44	3.03	0.20
Docosane	9	13.087	26.44	20.91	24.14	23.21	22.40	16.40	14.31	29.54	25.10	22.31	20.83	31.00	19.32	20.72	18.56	19.81
11-Methyldocosane	10	13.35	0.24	0.13	0.00	0.00	0.20	0.16	0.20	0.00	0.34	0.21	0.00	0.58	0.28	0.40	0.14	0.00
10-Methyldocosane	11	13.58	1.01	0.94	1.63	0.89	1.51	1.02	1.65	0.98	1.18	3.15	1.61	2.80	1.47	1.65	1.17	0.76
9-Methyldocosane	12	13.699	1.15	1.33	1.54	1.16	1.48	1.31	1.19	1.16	1.39	1.34	1.39	2.01	1.32	1.88	1.31	0.99
7-Methyldocosane	13	13.894	0.18	0.20	0.26	0.00	0.33	0.28	0.28	0.17	0.36	0.39	0.36	0.41	0.52	0.44	0.27	0.00
6-Methyldocosane	14	13.996	2.00	1.78	2.12	1.60	2.35	1.91	1.16	1.98	1.92	1.35	1.66	2.05	1.29	2.38	1.46	1.36
4-Methyldocosane	16	14.846	0.56	0.25	0.25	0.56	0.00	0.46	0.51	0.13	0.20	0.32	0.29	0.32	0.00	0.29	0.30	0.48
Tricosene	18	15.559	2.58	0.93	2.81	1.14	2.02	0.98	1.18	2.34	2.47	2.44	1.83	2.13	2.45	2.48	2.64	0.97
Tricosane	20	15.857	12.46	6.19	9.88	12.03	13.33	6.17	6.75	10.79	13.14	14.47	12.55	9.58	15.76	8.28	8.18	10.27
9+11-Methyltricosane	21	16.383	2.88	2.46	3.78	3.10	3.14	2.51	2.29	2.07	3.11	2.60	2.97	2.46	1.40	3.51	3.60	2.65
7-Methyltricosane	22	16.485	1.41	1.05	1.37	1.39	1.80	1.36	3.60	0.97	1.31	1.29	1.62	1.19	1.52	1.35	1.05	1.18
6-Methyltricosane	23	16.63	0.85	0.86	0.92	0.85	0.86	0.84	0.66	0.75	0.69	0.60	0.79	0.67	0.52	0.88	0.69	0.72
Mono-methyl branched tricosane	24	16.85	0.27	0.27	0.55	0.31	0.33	0.43	0.17	0.25	0.26	0.26	0.33	0.28	0.21	0.36	0.37	0.39
Tetracosane	25	17.029	3.34	2.05	2.59	2.61	3.81	2.70	1.78	2.89	2.56	2.47	2.97	2.32	1.87	2.89	2.04	2.48
Tetracosane	26	17.496	0.38	0.28	0.32	0.53	0.42	0.39	0.54	0.27	0.43	0.62	0.52	0.38	0.58	0.44	0.38	0.35
10+8-Methyltetracosane	27	17.581	0.47	0.50	0.51	0.74	0.67	0.67	0.56	0.42	0.47	0.62	0.74	0.60	0.75	0.71	0.59	0.61
Mono-methyl branched tetracosane	28	18.159	0.51	0.16	0.00	0.70	0.30	0.77	0.23	0.00	0.00	0.13	0.27	0.00	0.00	0.00	0.00	0.70
Mono-methyl branched tetracosane	29	18.277	0.24	0.09	0.31	0.00	0.00	0.00	0.00	0.19	0.17	0.23	0.00	0.33	0.00	0.36	0.43	0.00
Pentacosene	30	18.736	2.06	1.02	2.39	1.15	1.71	1.15	1.03	1.95	2.17	1.69	1.84	1.69	1.24	1.91	2.51	1.06
Pentacosene	31	18.898	1.68	0.60	1.53	0.88	1.17	0.61	0.74	1.46	1.33	1.45	1.22	1.25	1.12	1.44	1.76	0.56
Pentacosane	33	19.246	4.10	2.40	3.55	4.46	3.99	2.67	0.94	2.75	3.84	5.71	4.75	2.75	9.80	3.30	3.37	2.45
9+11-Methylpentacosane	34	19.815	2.62	3.79	3.83	4.54	2.54	4.22	4.16	2.00	2.92	1.76	3.24	2.16	0.98	3.46	3.56	3.87
9-Methylpentacosane	35	19.943	0.50	0.95	0.69	1.03	0.61	1.23	0.62	0.62	0.00	0.41	0.97	0.56	0.20	0.69	0.54	1.13
5-Methylpentacosane	36	20.147	0.00	1.02	0.59	1.07	1.03	1.36	0.00	0.59	0.61	0.56	1.21	0.54	0.00	0.59	0.59	1.25
4-Methylpentacosane	37	20.283	0.94	0.32	0.00	0.00	0.00	0.39	0.43	0.00	0.00	0.00	0.00	0.39	0.00	0.77	0.14	0.36
2-Methylpentacosane	38	20.393	0.53	0.49	0.52	0.54	0.00	0.46	0.82	0.38	0.32	0.50	0.51	0.00	0.00	0.00	0.64	0.42
Hexacosane	39	20.546	2.58	2.59	2.14	2.31	2.93	4.03	1.86	2.41	1.67	2.15	2.78	1.87	2.18	2.78	1.86	3.70
6-Methylhexacosane	40	20.648	0.00	0.00	0.00	1.01	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.68	0.00
Mono-methyl branched hexacosane	41	21.073	0.26	0.17	0.00	0.31	0.17	0.27	0.57	0.13	0.22	0.18	0.25	0.23	0.35	0.24	0.22	0.25
4-Methylhexacosane	42	21.192	0.80	0.91	0.50	1.13	1.25	1.56	1.65	0.86	1.33	1.16	1.55	1.84	2.14	2.53	0.83	1.44
Heptacosene	43	21.48	0.31	0.35	0.19	0.53	0.00	0.49	0.52	0.00	0.20	0.00	0.18	0.40	0.00	0.00	0.36	0.45
11+13-Methylheptacosane	44	21.778	0.29	0.00	0.00	0.00	0.33	0.00	0.48	0.00	0.26	0.00	0.00	0.00	1.02	0.28	0.40	0.00
10-Methylheptacosane	45	21.956	0.29	0.00	0.47	0.00	0.00	0.62	0.00	0.47	0.33	0.00	0.00	0.44	0.00	0.44	0.69	0.57
7-Methylheptacosane	46	22.007	0.42	0.00	0.00	0.00	0.00	0.22	1.35	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.14	0.20
Nonacosene	47	22.44	3.10	2.58	2.03	1.87	3.21	2.91	4.00	2.39	2.91	1.84	2.40	2.01	5.62	1.59	2.01	2.67
Nonacosane	48	22.942	1.50	1.28	1.47	1.76	1.58	1.49	1.78	0.86	1.42	2.72	1.66	0.97	5.73	1.01	1.21	1.37
11+13-Methylnonacosane	49	23.511	0.95	2.94	1.65	3.16	1.13	3.82	3.14	1.07	1.05	0.87	1.56	1.02	0.05	1.52	1.52	3.51
7-Methylnonacosane	51	23.672	0.51	1.00	0.54	0.91	0.55	1.47	0.87	0.48	0.42	0.37	0.63	0.32	0.00	0.56	0.47	1.35
5-Methylnonacosane	52	23.842	0.52	1.19	0.51	1.18	0.58	1.85	0.86	0.51	0.38	0.57	0.69	0.27	0.00	0.40	0.67	1.70
Mono-methyl branched nonacosane	53	24.309	0.55	1.09	0.70	1.43	1.09	2.19	1.39	0.53	0.63	1.03	0.82	0.41	0.27	1.01	0.93	2.01
Mono-methyl branched nonacosane	58	25.371	0.00	0.06	0.00	0.19	0.00	0.22	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.20
Mono-methyl branched triacontane	59	25.719	0.00	0.46	0.39	0.32	0.00	0.56	0.85	0.47	0.27	0.10	0.00	0.00	0.00	0.42	0.61	0.52
Hentriacontene	60	26.017	2.53	6.47	5.02	2.85	3.35	6.08	6.42	5.20	3.55	2.60	3.77	4.10	1.56	4.90	4.51	5.58
Hentriacontene	61	26.246	1.39	1.52	1.34	1.00	1.11	1.40	1.35	1.68	1.08	1.01	1.15	0.96	0.99	1.32	1.70	1.28
Hentriacontene	62	26.416	1.58	1.34	1.29	0.46	1.06	0.00	1.02	1.83	1.26	1.35	0.89	1.00	0.88	1.37	2.07	0.00
Hentriacontane	63	26.722	1.16	0.76	0.86	1.31	1.02	0.41	0.94	0.62	1.17	2.60	1.21	0.59	4.69	0.84	0.87	0.38
9+11-Methylhentriacontane	64	27.283	0.66	2.09	1.03	1.75	0.74	2.42	2.64	0.42	0.62	0.65	0.88	0.76	0.00	1.16	1.04	2.22
6-Methylhentriacontane	66	27.444	0.37	1.56	0.43	1.20	0.64	1.66	1.16	0.48	0.14	0.40	0.46	0.35	0.00	0.52	0.44	1.52
5-Methylhentriacontane	67	28.651	0.07	0.00	0.00	0.16	0.00	0.13	0.33	0.00	0.00	0.00	0.00	0.12	0.00	0.11	0.13	0.12
4-Methylhentriacontane	68	29.007	0.00	0.52	0.35	0.00	0.22	0.00	1.12	0.45	0.31	0.03	0.00	0.36	0.00	0.00	0.52	0.00
Tritriacontene	69	29.305	2.24	6.98	4.54	2.87	4.05	5.04	5.16	4.02	2.79	2.51	4.53	3.66	1.53	3.94	4.81	4.63
Tritriacontene	70	29.424	1.92	0.00	0.00	0.00	0.00	0.00	0.00	1.63	1.05	1.76	0.00	1.76	1.29	1.17	1.51	0.00
Tritriacontene	71	29.509	0.00	0.00	0.72	0.53	0.59	0.70	1.00	0.98	0.71	0.00	0.00	0.00	0.57	0.84	0.96	0.65
Tritriacontene	72	29.627	0.75	0.92	0.53	0.71	0.91	0.79	0.95	0.00	0.90	1.29	0.76	0.00	1.03	0.00	0.79	0.72
8-Methyltritriacontane	73	29.738	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.00	0.58	0.00	0.00	0.00
7-Methyltritriacontane	74	29.891	0.98	3.12	0.81	2.42	0.95	2.12	3.10	0.80	1.59	0.88	1.14	1.19	0.42	1.66	1.66	1.95
6-Methyltritriacontane	75	30.095	0.00	0.59	0.00	0.38	0.40	0.59	0.52	0.00	0.00	0.00	0.44	0.00	0.00	0.23	0.18	0.54
Mono-methyl branched tritriacontane	76	30.145	0.46	0.68	0.00	0.53	0.33	0.67	1.56	0.00	0.00	0.30	0.27	0.00	0.00	0.36	0.35	0.61
4-Methyltritriacontane	77	30.477	0.00	0.19	0.00	0.18	0.00	0.26	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.17	0.24
2-Methyltritriacontane	78	30.621	0.11	0.36	0.00	0.20	0.00	0.33	0.75	0.00	0.00	0.28	0.00	0.00	0.00	0.29	0.00	0.31
6-Methylytetratriacontane	79	30.664	0.00	0.38	0.74	0.19	0.85	0.37	1.14	0.54	0.49	0.17	0.00	0.43	0.00	0.32	0.58	0.34
6-Methyltetratriacontane	80	30.74	0.13	0.64	0.00	0.34	0.00	0.56	0.00	0.42	0.37	0.31	1.13	0.37	0.00	0.47	0.65	0.51
Pentatriacontene	81	30.825	1.94	3.94	2.05	2.03	2.67	2.16	2.24	3.18	2.64	2.83	3.22	2.88	2.31	3.09	3.94	1.98
Pentatriacontane	82	30.978	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00
18-Methylpentatriacontane	83	31.012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00
Mono-methyl branched pentatriacontane	84	31.267	0.28	1.15	0.08	0.48	0.70	0.95	0.91	0.82	0.35	0.54	0.77	0.34	0.00	0.44	0.35	0.88
Mono-methyl branched pentatriacontane	85	31.862	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.35	0.00

Table 42: A breakdown of the hydrocarbon types analysed at sampling day 5.

Day 5	Percentage
Alkenes	12.3
Alkanes	12.3
Mono-methyl branched Alkanes	75.4

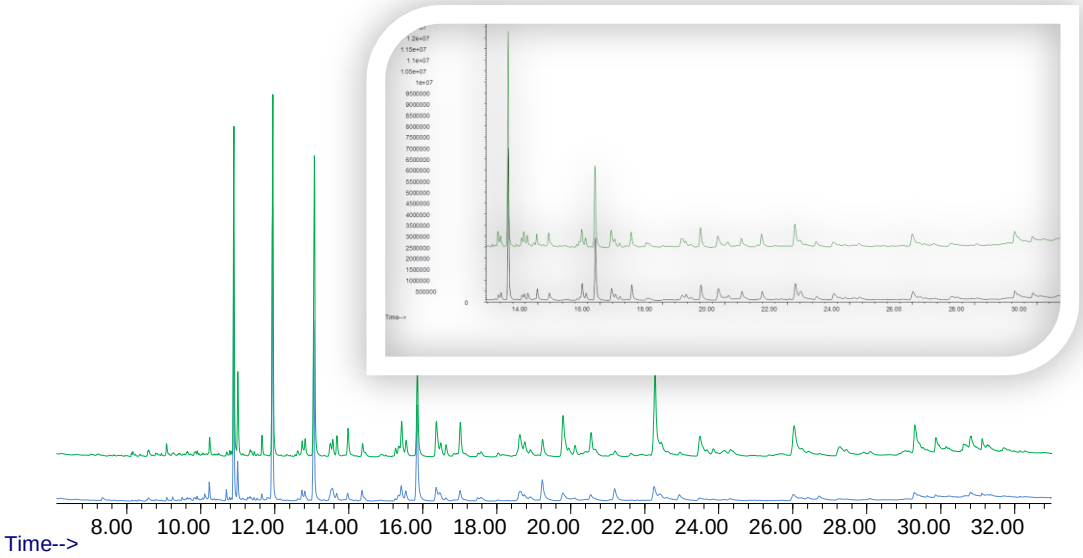


Figure 184: Day 5 control and paracetamol diet chromatograms for comparison.

6.3.9 Day 6

At sampling day 6, 55 hydrocarbons were detected (Table 43), 72.7% of those were branched alkanes and ranged between 21 and 35 in chain length (Table 44). No obvious chemical changes were observed in relation to the compounds identified (Figure 185).

Table 43: List of all hydrocarbons identified at sampling day 6 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	5EAPB	6+5APB	6APB	AMT	Benzofury Beige	Benzofury Blue	Benzocaine	Benzofury Green	Blow	Caffiene	Ivory Wave	Paracetamol	Pink Panther	Synthacaine	MDA
9+11-Methylhenicosane	1	12.152	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.87	1.06	0.00	0.00	1.36	0.00	0.00
Mono-methyl branched henicosane	3	12.339	0.00	0.15	0.00	0.22	0.14	0.00	0.00	0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.00	0.20
7-Methylhenicosane	5	12.56	0.00	0.19	0.00	0.37	0.12	0.00	0.00	0.09	0.00	0.26	0.45	0.00	0.37	0.71	0.11	0.33
Docosene	7	12.73	0.00	1.49	2.99	0.92	1.42	0.59	0.38	2.19	1.70	1.74	2.01	1.45	0.87	2.42	1.86	0.83
Docosene	8	12.815	0.00	0.80	2.16	0.94	1.60	0.76	0.77	2.04	2.36	1.29	2.41	1.58	1.26	2.08	2.70	0.85
Docosane	9	13.087	7.35	11.82	16.77	27.38	22.56	14.77	11.86	17.07	19.68	9.23	12.99	16.29	16.65	6.08	16.52	24.74
11-Methyldocosane	10	13.35	0.69	0.41	0.25	0.19	0.00	0.00	0.00	0.31	0.00	0.43	0.94	0.31	0.29	1.48	0.27	0.17
10-Methyldocosane	11	13.58	3.07	1.04	1.80	1.20	1.24	1.53	1.34	1.44	1.89	1.57	3.82	0.91	1.59	2.92	1.68	1.08
9-Methyldocosane	12	13.699	2.39	1.37	1.89	1.43	1.59	1.60	2.07	1.50	1.81	1.90	3.07	1.44	1.82	2.91	1.96	1.29
7-Methyldocosane	13	13.894	0.97	0.71	0.43	0.26	0.00	0.00	0.00	0.30	0.00	0.80	2.35	0.27	0.00	3.26	0.34	0.24
6-Methyldocosane	14	13.996	3.17	2.09	2.37	1.98	2.46	2.12	1.74	1.76	2.37	1.97	2.85	1.56	1.88	3.45	1.81	1.79
5-Methyldocosane	15	14.208	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.29	0.00	0.00	1.84	0.00	0.00
4-Methyldocosane	16	14.846	0.00	0.49	0.52	0.40	0.21	0.00	0.00	0.00	0.00	3.69	0.62	0.20	0.00	0.00	0.32	0.36
Tricosene	18	15.559	1.58	0.60	1.76	1.15	1.65	0.99	0.88	1.37	2.00	1.25	1.84	0.99	1.58	2.41	2.06	1.04
Tricosane	20	15.857	3.82	2.40	5.14	9.27	7.44	4.14	4.05	4.50	7.21	3.87	4.80	3.84	6.07	4.61	5.44	8.38
9+11-Methyltricosane	21	16.383	4.92	3.57	5.86	4.15	4.15	3.77	4.05	2.20	5.70	4.57	5.10	3.35	4.11	6.61	4.11	3.75
7-Methyltricosane	22	16.485	3.37	1.09	1.22	1.30	1.23	1.62	1.35	1.10	1.71	2.28	3.94	0.84	1.86	3.79	1.16	1.18
6-Methyltricosane	23	16.63	1.27	0.68	0.87	1.00	0.99	1.12	1.39	0.66	1.32	0.91	1.23	0.64	1.11	0.00	0.83	0.90
Mono-methyl branched tricosane	24	16.85	1.10	0.37	0.38	0.22	0.16	0.56	0.46	0.48	0.00	1.85	1.18	0.33	0.59	1.19	0.48	0.20
Tetracosane	25	17.029	2.57	1.58	1.79	2.49	2.45	2.61	2.74	1.38	2.73	2.32	2.74	1.35	2.63	2.91	2.16	2.25
Tetracosane	26	17.496	3.37	0.00	0.40	0.45	0.37	0.60	0.00	0.39	0.26	0.75	1.37	0.49	0.70	3.30	0.38	0.40
10+8-Methyltetracosane	27	17.581	2.17	1.16	0.88	0.51	0.67	1.01	1.25	0.48	0.00	1.31	2.55	0.31	1.19	2.92	0.88	0.46
Mono-methyl branched tetracosane	28	18.159	0.00	0.00	0.00	0.42	0.00	0.00	0.68	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.48	0.38
Pentacosene	30	18.736	2.52	0.00	1.55	1.37	1.52	1.01	1.24	0.87	1.44	1.20	3.03	0.85	1.44	0.00	1.60	1.24
Pentacosene	31	18.898	0.54	0.00	0.75	0.66	1.16	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.00	0.00	0.97	0.60
Pentacosane	33	19.246	4.28	0.77	1.75	2.17	2.03	2.04	2.63	2.74	4.31	1.84	2.08	2.09	3.37	4.79	3.02	1.96
9+11-Methylpentacosane	34	19.815	3.57	5.34	5.97	4.64	5.06	5.05	8.30	2.00	7.13	4.57	4.36	3.74	4.56	4.94	4.16	4.20
9-Methylpentacosane	35	19.943	0.73	0.95	0.72	0.83	1.01	1.11	1.73	0.61	0.92	1.00	0.66	0.59	0.89	0.00	0.66	0.75
5-Methylpentacosane	36	20.147	0.00	0.77	0.67	1.04	1.26	0.70	1.81	0.41	0.00	1.61	0.00	0.45	0.90	0.00	0.52	0.94
2-Methylpentacosane	38	20.393	0.00	0.54	0.70	0.90	0.96	0.00	1.20	0.00	0.00	1.05	0.00	0.49	0.96	0.00	0.59	0.81
Hexacosane	39	20.546	0.78	1.75	1.94	1.78	2.64	1.36	3.84	2.13	1.08	2.78	1.92	1.57	3.19	0.00	2.39	1.61
6-Methylhexacosane	40	20.648	0.00	1.23	1.18	0.72	1.14	0.00	2.39	0.00	0.00	1.37	0.00	0.66	1.30	0.00	0.85	0.65
Mono-methyl branched hexacosane	41	21.073	2.90	0.36	0.23	0.29	0.41	0.00	0.62	0.24	0.00	0.67	0.90	0.39	0.60	2.91	0.30	0.26
4-Methylhexacosane	42	21.192	4.17	1.15	0.72	0.81	1.22	1.02	1.07	0.97	1.08	2.32	3.17	0.87	1.81	6.94	0.59	0.73
Heptacosene	43	21.48	0.78	0.92	0.48	0.31	0.42	0.66	1.05	0.00	0.00	0.88	0.00	0.24	0.60	0.00	0.33	0.28
10-Methylheptacosane	45	21.956	0.00	0.56	0.37	0.24	0.24	0.00	0.51	0.00	0.00	3.34	0.00	0.41	0.00	0.00	0.59	0.21
Nonacosene	47	22.44	1.19	0.00	0.95	1.63	1.99	2.14	3.52	1.78	1.67	1.58	1.86	1.98	1.83	0.00	1.77	1.47
Nonacosane	48	22.942	3.19	0.61	1.36	0.87	0.34	1.40	2.33	2.59	2.69	1.23	1.30	2.09	2.03	4.08	1.55	0.79
11+13-Methylnonacosane	49	23.511	4.63	5.25	3.87	2.72	2.68	5.00	6.12	1.97	4.91	3.19	2.91	2.73	2.94	2.22	2.53	2.46
7-Methylnonacosane	51	23.672	1.21	0.99	0.78	0.64	0.80	1.58	2.03	0.77	0.81	0.71	0.00	0.65	0.81	0.00	0.91	0.57
5-Methylnonacosane	52	23.842	0.00	0.77	0.55	0.63	0.76	1.30	1.60	0.81	0.00	0.70	0.00	0.71	1.18	0.00	1.24	0.57
Mono-methyl branched nonacosane	53	24.309	2.04	0.51	1.05	0.80	1.02	1.80	2.47	1.62	0.00	0.94	0.91	0.76	1.30	0.00	1.80	0.72
Mono-methyl branched triacontane	59	25.719	0.00	1.10	0.85	0.35	0.45	0.00	0.55	0.30	0.00	1.46	0.00	0.91	0.00	0.00	0.36	0.32
Hentriacontene	60	26.017	4.72	8.75	6.84	5.79	5.71	7.11	5.33	7.49	7.97	3.73	5.04	8.74	6.92	3.21	6.29	5.21
Hentriacontene	61	26.246	1.14	1.19	1.67	0.75	1.20	1.56	0.96	2.21	0.84	0.59	0.21	2.02	1.28	0.00	2.21	1.54
Hentriacontene	62	26.416	0.00	0.60	1.41	0.34	1.01	0.94	0.00	1.90	0.00	0.00	0.65	1.87	0.77	0.00	2.55	1.32
Hentriacontane	63	26.722	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.10	0.00	0.00	0.00	1.33	0.00	1.52	1.05	1.46
9+11-Methylhentriacontane	64	27.283	3.58	4.15	2.65	1.72	1.47	3.63	2.85	1.60	1.99	0.92	1.64	2.38	0.69	0.24	1.91	1.11
6-Methylhentriacontane	66	27.444	0.97	1.64	0.93	0.59	0.80	2.05	1.13	0.87	0.00	1.04	0.00	0.80	0.00	0.00	0.77	0.61
5-Methylhentriacontane	67	28.651	0.51	0.26	0.20	0.11	0.37	0.00	0.00	0.14	0.00	0.41	0.00	0.23	0.00	0.00	0.07	0.10
4-Methylhentriacontane	68	29.007	1.11	1.36	0.73	0.53	0.12	0.87	0.62	0.62	0.00	0.99	0.47	1.19	0.44	0.00	0.40	0.43
Tritriacontene	69	29.305	3.52	7.70	3.77	4.13	3.61	7.15	3.31	6.55	6.10	4.14	2.90	5.97	7.08	3.68	5.28	4.56
Tritriacontene	70	29.424	1.07	0.00	0.82	0.00	1.46	0.00	0.00	2.84	0.00	1.10	0.66	2.41	0.00	1.92	1.15	1.97
Tritriacontene	71	29.509	0.91	1.23	0.52	0.27	0.00	1.01	0.26	1.35	1.13	0.82	0.00	1.36	0.73	0.00	0.50	0.94
Tritriacontene	72	29.627	0.00	0.86	0.00	0.14	0.00	0.00	0.00	1.60	0.00	0.00	0.00	1.33	0.43	1.95	0.00	1.11
7-Methyltritriacontane	74	29.891	3.24	4.95	1.91	1.91	2.16	3.46	2.51	1.85	2.94	2.07	1.74	2.85	1.44	3.26	1.37	1.29
6-Methyltritriacontane	75	30.095	1.08	0.54	0.05	0.14	0.18	0.62	0.35	0.00	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.00
Mono-methyl branched tritriacontane	76	30.145	0.62	1.91	0.12	0.25	0.41	1.08	0.40	0.62	0.00	1.38	0.00	0.60	0.00	0.00	0.08	0.43
2-Methyltritriacontane	78	30.621	1.70	2.35	0.35	1.07	0.49	0.36	0.25	0.00	0.00	1.76	0.65	0.53	0.00	0.00	0.30	0.00
6-Methyltetratriacontane	79	30.664	0.00	0.00	1.09	0.00	0.19	1.07	0.40	0.83	0.00	0.00	1.13	0.55	0.76	0.00	0.26	0.58
6-Methyltetratriacontane	80	30.74	0.00	0.00	0.00	0.00	0.37	0.00	0.00	0.68	0.00	0.00	0.00	1.01	0.61	0.00	0.48	0.47
Pentatriacontene	81	30.825	1.17	4.96	2.32	2.18	2.02	3.42	1.49	5.59	2.25	3.56	2.65	5.53	4.68	2.08	2.99	3.89
Mono-methyl branched pentatriacontane	84	31.267	0.33	0.86	0.68	0.42	0.86	1.70	0.12	0.87	0.00	0.92	0.00	0.70	0.00	0.00	0.05	0.61
Mono-methyl branched pentatriacontane	85	31.862	0.00	0.83	0.00	0.00	0.00	0.00	0.00	0.57	0.00	0.23	0.00	0.29	0.00	0.00	0.00	0.39

Table 44: A breakdown of the hydrocarbon types analysed at sampling day 6.

Day 6	Percentage
Alkenes	14.5
Alkanes	12.7
Mono-methyl branched Alkanes	72.7

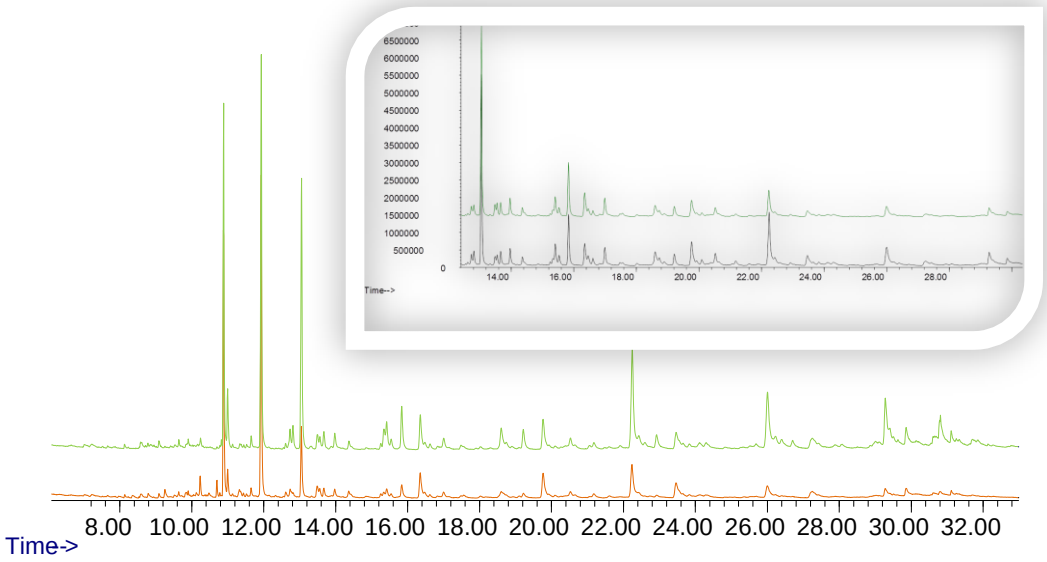


Figure 185: Day 6 control and 5EAPB diet chromatograms for comparison.

Principle component analysis of day 5 and 6

Principle component analysis of day 5 and 6 shows PC 1 and 2 plotted displaying 89% of data variance. Previous analysis concerning all sampling days showed clear separation of these days with minimal overlap. A closer look shows two clusters but with 2 treatments appearing to cluster with the incorrect sampling day. 6APB and MDA cluster to the left of the plot instead of to the right with all other day 6 samples.

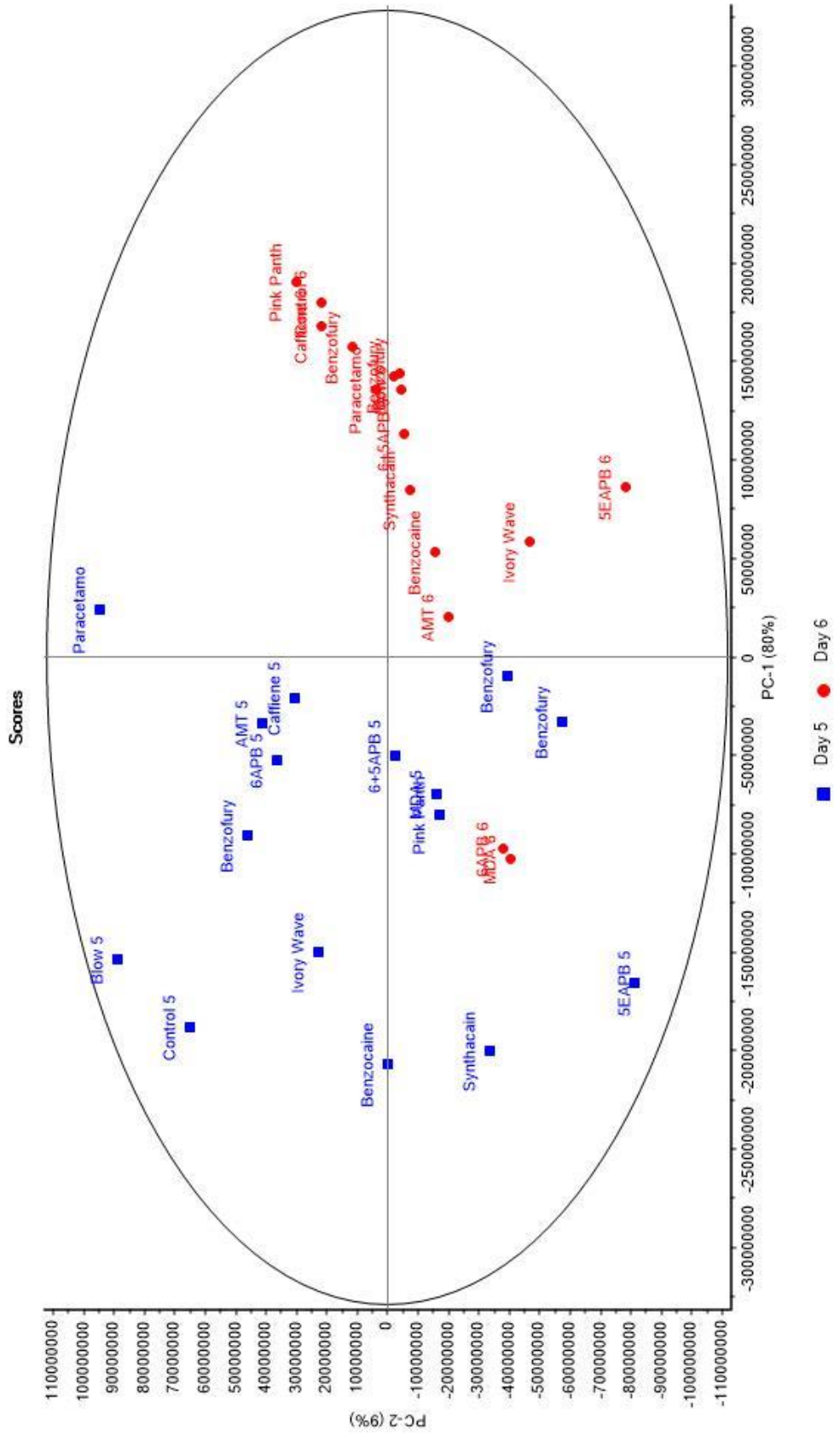


Figure 186: PCA plot of development days 5 and 6 analysed using identified hydrocarbons and peak areas.

6.3.10 Day 7

Analysis of day 7 peaks showed 52 hydrocarbons identified (Table 45), 73.1% of these were mono-methyl alkanes (Table 46). They ranged between 21 and 35 in chain length, no real differences were observed in the hydrocarbons present between the different sample populations (Figure 187).

Table 45: List of all hydrocarbons identified at sampling day 7 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	5EAPB	6+5APB	6APB	AMT	Benzofury Beige	Benzofury Blue	Benzocaine	Benzofury Green	Blow	Caffiene	Ivory Wave	Paracetamol	Pink Panther	Synthacaine	MDA
9+11-Methylhenicosane	1	12.152	0.00	0.39	0.00	0.00	0.00	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mono-methyl branched henicosane	3	12.339	0.18	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.00	0.23	0.87	0.00	0.32	0.00	0.00	0.00
7-Methylhenicosane	5	12.56	0.08	0.25	0.00	0.00	0.00	0.19	0.15	0.00	0.20	0.00	0.00	0.00	0.20	0.00	0.00	0.00
Docosene	7	12.73	2.40	0.53	4.22	0.84	3.81	1.25	0.80	0.00	0.73	0.88	0.31	2.13	0.97	8.28	3.02	1.14
Docosene	8	12.815	1.30	0.81	2.52	0.00	2.64	1.08	1.65	1.98	0.98	1.07	0.00	1.24	0.75	3.61	3.16	0.00
Docosane	9	13.087	12.14	13.98	6.65	7.33	10.43	13.18	21.01	12.28	10.59	9.78	10.48	11.79	19.59	15.64	12.83	9.92
11-Methyldocosane	10	13.35	0.19	0.62	0.43	0.60	0.50	0.00	0.00	0.00	0.61	0.28	0.30	0.39	0.32	0.00	0.00	0.82
10-Methyldocosane	11	13.58	2.03	1.95	2.21	2.14	0.00	3.24	1.93	0.94	0.99	1.99	3.68	1.09	2.09	3.27	2.08	2.90
9-Methyldocosane	12	13.699	1.61	1.97	3.87	2.04	5.16	3.15	2.55	1.18	1.45	2.25	2.91	1.61	1.86	3.15	2.20	2.76
7-Methyldocosane	13	13.894	0.32	1.20	1.45	1.50	0.00	0.60	0.32	0.00	1.12	0.66	0.76	0.77	0.67	0.00	0.66	2.03
6-Methyldocosane	14	13.996	2.09	2.26	2.87	2.52	2.74	4.61	2.05	1.45	1.59	2.21	2.98	1.81	2.24	3.21	2.80	3.41
4-Methyldocosane	16	14.846	0.50	0.63	0.00	0.00	1.35	0.59	0.71	0.00	0.00	0.51	0.99	0.00	0.20	0.00	0.00	0.00
Tricosene	19	15.721	0.00	0.00	0.00	0.85	0.00	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.00	0.00	0.00	1.15
Tricosane	20	15.857	5.32	6.97	2.59	6.52	6.00	6.17	7.38	5.95	5.03	6.05	5.96	4.91	13.87	10.06	3.64	8.82
9+11-Methyltricosane	21	16.383	4.12	8.30	4.15	4.87	8.59	5.63	4.92	3.36	2.42	3.84	9.19	2.53	4.43	8.18	3.78	6.58
7-Methyltricosane	22	16.485	2.11	1.86	2.07	2.27	3.93	2.26	2.28	0.85	2.59	1.96	4.40	0.88	2.54	2.03	1.64	3.07
6-Methyltricosane	23	16.63	1.08	0.00	0.00	1.37	0.00	1.95	1.08	0.00	0.68	1.02	1.58	0.56	1.12	1.23	1.08	1.86
Mono-methyl branched tricosane	24	16.85	0.39	1.11	1.02	1.26	0.81	0.74	0.33	0.00	0.84	0.94	0.91	0.33	0.59	0.00	0.73	1.70
Tetracosane	25	17.029	2.59	2.37	0.00	2.80	2.77	5.34	2.53	1.66	0.00	3.08	3.44	0.88	3.43	2.99	2.24	3.79
Tetracosane	26	17.496	0.39	0.00	0.91	1.68	1.02	0.73	0.40	0.24	1.14	1.21	1.02	1.01	1.04	0.97	0.00	0.99
10+8-Methyltetracosane	27	17.581	0.83	2.83	2.16	2.16	4.07	1.78	0.89	0.00	1.13	1.93	1.66	0.98	1.19	1.08	1.42	3.97
Mono-methyl branched tetracosane	28	18.159	0.52	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	1.00	0.00	0.40	0.00	0.00	0.00
Pentacosene	30	18.736	1.47	0.70	1.28	1.19	0.00	2.01	1.66	0.00	1.10	0.00	0.00	0.60	1.53	2.36	1.89	0.00
Pentacosene	31	18.898	0.00	0.50	0.00	1.10	0.00	0.50	1.04	0.00	0.38	0.81	0.00	0.00	0.93	0.00	0.00	0.00
Pentacosane	33	19.246	2.17	2.24	1.83	6.49	3.45	3.48	3.10	5.58	6.16	5.33	3.53	5.67	8.29	9.07	2.40	3.37
9+11-Methylpentacosane	34	19.815	3.21	9.58	2.27	6.75	6.47	6.14	5.61	6.27	2.79	4.26	6.59	3.01	3.42	3.37	3.73	6.31
9-Methylpentacosane	35	19.943	0.91	0.00	0.00	0.00	0.00	2.10	0.88	0.00	0.34	0.91	1.40	0.76	0.76	0.00	1.05	0.00
5-Methylpentacosane	36	20.147	0.79	0.69	0.00	1.20	1.42	1.59	0.87	0.66	0.43	1.39	1.31	0.00	1.12	0.00	0.00	1.38
2-Methylpentacosane	38	20.393	0.50	0.56	0.00	0.83	0.00	0.79	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hexacosane	39	20.546	2.78	2.38	0.91	3.02	1.96	7.11	2.58	2.00	1.80	3.84	2.54	2.97	2.86	1.93	2.13	1.91
6-Methylhexacosane	40	20.648	0.00	1.77	0.00	0.00	0.00	0.00	1.15	0.00	0.78	0.00	1.56	0.00	0.00	0.00	0.00	0.00
Mono-methyl branched hexacosane	41	21.073	0.25	0.68	0.86	1.19	0.73	0.59	0.36	0.76	1.07	1.01	1.21	0.96	0.59	0.52	0.00	0.71
4-Methylhexacosane	42	21.192	0.89	1.55	6.04	4.56	5.12	1.61	0.89	1.04	6.78	3.50	9.22	2.50	1.95	1.17	1.73	5.00
11+13-Methylheptacosane	44	21.778	0.28	0.81	0.00	0.97	0.00	0.72	0.49	0.00	0.35	0.88	0.67	0.59	0.30	0.00	0.00	0.00
Nonacosene	47	22.44	1.59	1.64	2.02	1.74	2.36	3.13	1.89	1.67	0.00	3.54	1.00	1.13	0.00	0.00	3.13	2.30
Nonacosane	48	22.942	1.69	1.34	3.06	5.53	2.76	1.91	1.28	6.08	5.96	4.18	2.98	5.10	4.67	4.20	2.30	2.70
11+13-Methylnonacosane	49	23.511	2.28	5.88	2.90	5.51	3.54	3.62	2.29	4.95	2.78	4.15	4.07	3.23	1.67	1.27	2.66	3.45
7-Methylnonacosane	51	23.672	0.97	1.35	0.00	1.71	0.51	1.52	0.81	0.77	0.69	1.47	1.02	0.99	0.53	0.00	1.08	0.50
5-Methylnonacosane	52	23.842	1.28	0.78	0.00	0.43	0.00	1.27	0.73	0.67	0.64	1.12	1.19	0.71	0.73	0.00	0.69	0.00
Mono-methyl branched nonacosane	53	24.309	1.71	0.00	1.19	0.98	0.00	2.21	1.06	0.00	1.40	2.76	1.61	1.21	1.18	0.00	1.13	0.00
Mono-methyl branched triacontane	59	25.719	0.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00	0.00	0.00	1.18	0.00
Hentriacontene	60	26.017	9.26	2.91	7.77	3.10	3.18	1.60	3.16	12.57	4.97	4.21	1.23	6.76	1.80	2.07	9.36	3.11
Hentriacontene	61	26.246	1.83	0.81	2.01	1.21	0.00	0.24	1.27	2.42	1.12	1.19	0.00	1.68	0.67	0.00	2.78	0.00
Hentriacontene	62	26.416	1.17	0.00	0.00	0.00	0.00	0.00	2.13	2.06	0.77	0.00	0.00	1.25	0.00	0.00	2.89	0.00
Hentriacontane	63	26.722	1.29	0.00	0.00	0.00	1.73	0.00	0.75	1.81	1.08	1.09	1.18	1.29	2.75	2.91	0.75	1.69
9+11-Methylhentriacontane	64	27.283	2.28	4.58	1.46	2.05	2.74	1.10	1.46	2.47	2.14	1.36	2.29	2.15	0.70	0.00	0.35	2.67
4-Methylhentriacontane	68	29.007	0.16	0.00	0.00	0.33	0.00	0.00	0.13	0.00	0.80	0.17	0.00	0.00	0.11	0.00	0.00	0.00
Tritriacontene	69	29.305	7.79	2.23	7.21	2.06	2.10	1.06	3.01	8.79	3.82	2.25	0.34	5.32	1.29	2.39	6.55	2.05
Tritriacontene	70	29.424	0.00	1.04	1.78	1.05	0.88	0.43	1.00	0.00	1.35	0.91	0.30	1.87	0.69	0.75	2.18	0.86
Tritriacontene	71	29.509	1.01	0.73	1.92	0.00	0.80	0.44	0.93	1.35	0.90	0.00	0.00	1.15	0.10	0.00	1.16	0.79
Tritriacontene	72	29.627	1.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.58	0.28	2.00	0.66	0.98	0.00	0.00
8-Methyltritriacontane	73	29.738	0.00	0.00	0.00	0.92	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.06	0.00	0.00	0.00
7-Methyltritriacontane	74	29.891	3.27	3.40	4.61	1.49	2.70	1.64	2.17	3.24	4.75	1.79	1.23	2.36	0.69	1.23	1.39	2.63
6-Methyltritriacontane	75	30.095	0.24	0.00	0.83	0.65	1.16	0.00	0.33	0.00	0.00	1.04	0.31	1.36	0.41	0.00	0.00	1.13
Mono-methyl branched tritriacontane	76	30.145	0.62	1.00	1.33	0.00	0.00	0.43	0.48	0.00	0.81	0.37	0.00	0.00	0.00	0.00	0.00	0.00
2-Methyltritriacontane	78	30.621	0.43	0.00	0.00	0.00	0.00	0.00	0.22	1.93	1.63	1.36	0.00	2.42	0.32	0.00	1.76	0.00
6-Methyltetratriacontane	79	30.664	0.43	0.38	2.42	0.31	0.00	0.00	0.69	0.00	1.18	0.62	0.00	1.08	0.17	0.00	3.59	0.00
Pentatriacontene	81	30.825	0.69	1.14	5.47	2.16	1.21	0.13	2.74	3.01	3.06	2.89	0.00	3.77	1.25	1.47	0.00	1.18
18-Methylpentatriacontane	83	31.012	3.26	1.14	1.94	0.00	0.00	0.00	0.00	0.00	1.42	0.00	0.00	1.45	0.00	0.60	0.59	0.00
Mono-methyl branched pentatriacontane	84	31.267	1.08	0.17	1.78	0.70	1.38	0.14	0.40	0.00	1.19	1.13	0.46	1.75	0.00	0.00	0.25	1.35

Table 46: A breakdown of the hydrocarbon types analysed at sampling day 7.

Day 7	Percentage
Alkenes	13.5
Alkanes	13.5
Mono-methyl branched Alkanes	73.1

Figure 187: Day 7 control and 6APB diet chromatograms for comparison.

6.3.11 Day 8

Analysis of day 8 components showed only 8 remaining populations due to early pupariation caused by drug presence. 49 hydrocarbons were identified within the samples on this day (Table 47) and all ranged between 21 and 35 in chain length. 71.4% of the hydrocarbons identified were mono-methyl alkanes (Table 48). No obvious differences were observed in the remaining samples in relation to hydrocarbons present.

Table 47: List of all hydrocarbons identified at sampling day 8 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	5EAPB	6APB	AMT	Blow	Caffiene	Paracetamol	MDA
9+11-Methylhenicosane	1	12.152	1.00	0.00	0.00	0.00	2.10	0.43	0.00	0.00
Mono-methyl branched henicosane	3	12.339	0.00	0.27	0.50	0.00	0.00	0.00	0.00	0.60
7-Methylhenicosane	5	12.56	0.77	0.09	0.00	0.33	1.17	0.34	0.33	0.00
Docosene	7	12.73	5.11	0.55	1.48	3.54	3.04	2.23	2.25	1.76
Docosane	9	13.087	26.03	15.95	15.38	14.20	18.04	11.36	10.65	18.29
11-Methyldocosane	10	13.35	1.12	0.00	0.67	0.64	3.26	0.49	0.43	0.79
10-Methyldocosane	11	13.58	6.82	1.65	2.21	3.06	2.97	2.98	1.51	2.63
9-Methyldocosane	12	13.699	6.66	2.03	2.96	3.68	1.75	2.91	1.22	3.52
7-Methyldocosane	13	13.894	2.14	0.00	1.08	1.22	6.36	0.94	0.82	1.29
6-Methyldocosane	14	13.996	6.66	1.41	3.22	3.51	3.92	2.91	1.69	2.38
2-Methyldocosane	17	14.905	1.58	0.53	0.00	0.61	0.00	0.69	0.00	0.41
Tricosene	18	15.559	5.12	0.99	1.35	1.54	2.21	2.24	1.68	1.04
Tricosane	20	15.857	6.93	5.49	5.47	6.49	10.35	4.60	4.73	4.41
9+11-Methyltricosane	21	16.383	2.11	4.30	3.91	10.58	3.15	6.40	2.48	7.19
7-Methyltricosane	22	16.485	1.87	1.54	3.25	2.44	2.78	3.89	1.39	1.66
6-Methyltricosane	23	16.63	0.00	0.89	1.31	1.70	0.00	1.35	0.81	1.16
Mono-methyl branched tricosane	24	16.85	0.00	0.46	0.56	0.82	0.00	0.74	0.66	0.56
Tetracosane	25	17.029	1.73	2.00	3.65	3.88	2.58	3.38	2.05	2.64
10+8-Methyltetracosane	27	17.581	2.73	0.98	2.08	1.77	4.07	1.92	1.40	1.20
Pentacosene	30	18.736	0.00	1.54	3.85	0.00	0.00	2.26	1.12	0.00
Pentacosane	33	19.246	5.47	3.10	3.12	5.05	8.16	4.86	4.19	3.43
9+11-Methylpentacosane	34	19.815	3.29	7.37	5.96	9.64	4.91	7.13	2.56	6.55
9-Methylpentacosane	35	19.943	0.00	1.19	1.67	1.64	0.00	1.20	0.00	1.12
2-Methylpentacosane	38	20.393	0.00	0.83	1.32	0.00	0.00	1.62	0.00	0.00
Hexacosane	39	20.546	0.00	3.25	5.71	3.75	0.00	3.67	2.25	2.55
6-Methylhexacosane	40	20.648	0.00	1.37	0.00	2.24	0.00	1.74	0.00	0.00
Mono-methyl branched hexacosane	41	21.073	1.33	0.38	0.77	0.00	1.99	0.89	0.62	0.92
4-Methylhexacosane	42	21.192	1.86	1.27	2.57	1.61	2.77	0.94	1.12	3.06
11+13-Methylheptacosane	44	21.778	0.00	0.70	0.92	0.72	0.00	0.00	0.00	1.09
10-Methylheptacosane	45	21.956	0.00	0.74	0.00	0.00	0.00	0.00	0.00	0.00
Nonacosene	47	22.44	0.00	2.72	0.30	0.00	0.00	2.17	0.33	0.36
Nonacosane	48	22.942	4.92	1.81	2.68	3.02	7.33	3.08	5.62	3.18
11+13-Methylnonacosane	49	23.511	1.92	4.39	4.34	5.16	2.87	3.45	2.35	5.16
7-Methylnonacosane	51	23.672	0.00	1.18	1.59	0.00	0.00	0.59	0.82	1.89
5-Methylnonacosane	52	23.842	0.00	1.31	0.91	0.00	0.00	0.31	1.19	1.09
Mono-methyl branched nonacosane	53	24.309	0.00	1.90	2.58	0.00	0.00	1.81	1.44	3.08
Mono-methyl branched triacontane	59	25.719	0.00	0.38	0.00	0.00	0.00	3.81	6.82	0.00
Hentriacontene	60	26.017	0.00	4.72	2.67	2.04	0.00	0.24	0.00	3.18
Hentriacontene	61	26.246	0.00	1.35	0.02	0.00	0.00	1.21	2.39	0.02
Hentriacontene	62	26.416	0.00	0.86	0.00	0.00	0.00	0.00	2.14	0.00
Hentriacontane	63	26.722	0.00	0.35	0.56	0.00	0.00	1.52	3.54	0.66
9+11-Methylhentriacontane	64	27.283	1.76	2.30	1.32	1.51	2.62	1.25	1.76	1.58
6-Methylhentriacontane	66	27.444	0.00	0.98	0.53	0.00	0.00	0.00	0.90	0.63
Tritriacontene	69	29.305	0.00	3.79	1.72	0.88	0.00	2.65	6.30	2.04
Tritriacontene	70	29.424	0.00	1.23	1.05	0.00	0.00	0.67	3.18	1.25
Tritriacontene	71	29.509	0.00	1.20	0.82	0.00	0.00	0.00	1.48	0.97
7-Methyltritriacontane	74	29.891	1.06	3.04	1.90	2.10	1.59	1.33	2.54	2.27
6-Methyltritriacontane	75	30.095	0.00	0.25	0.94	0.00	0.00	0.00	1.06	1.11
Mono-methyl branched tritriacontane	76	30.145	0.00	0.74	0.40	0.00	0.00	0.00	0.00	0.47
2-Methyltritriacontane	78	30.621	0.00	0.43	0.00	0.63	0.00	0.00	0.77	0.00
6-Methyltetratriacontane	79	30.664	0.00	0.77	0.00	0.00	0.00	0.00	1.70	0.00
Pentatriacontene	81	30.825	0.00	2.77	0.69	0.00	0.00	1.80	6.47	0.82
Mono-methyl branched pentatriacontane	84	31.267	0.00	0.67	0.00	0.00	0.00	0.00	1.23	0.00

Table 48: A breakdown of the hydrocarbon types analysed at sampling day 8.

Day 8	Percentage
Alkenes	14.3
Alkanes	14.3
Mono-methyl branched Alkanes	71.4

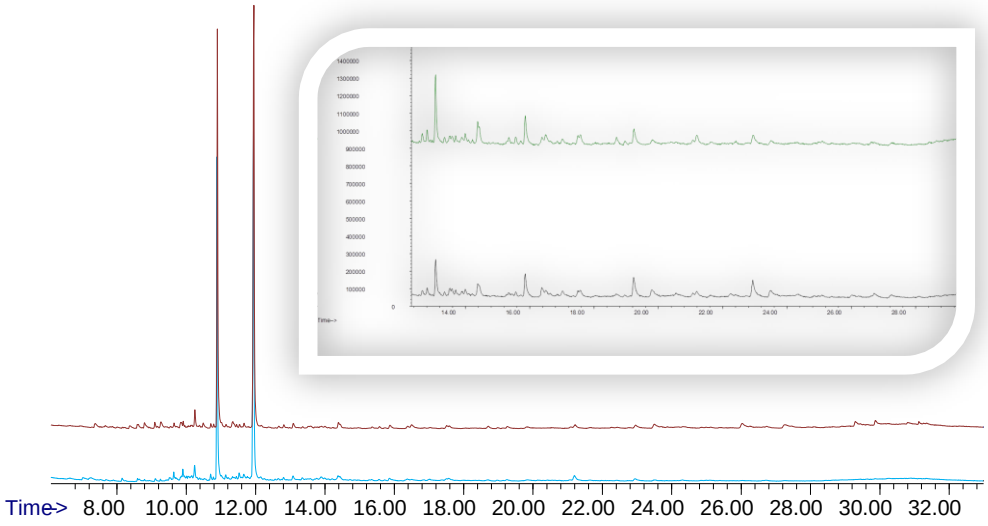


Figure 188: Day 8 control and AMT diet chromatograms for comparison.

Principle component analysis of day 7 and 8

Principle component analysis of day 7 and day 8 together showed clear separation with day 7 observed on the right of the plot and day 8 on the left. Although most samples appear to cluster together, the control sample of day 7 can be observed separate from the rest. In previous analysis of all sampling days together, day 7 and 8 could not be separated, analysis using principle component analysis of the two days has shown separation to be possible but further investigation into the source of the variation causing control to move away from the day 7 cluster is necessary to rule out the presence of drug triggering chemical changes within the hydrocarbon profile.

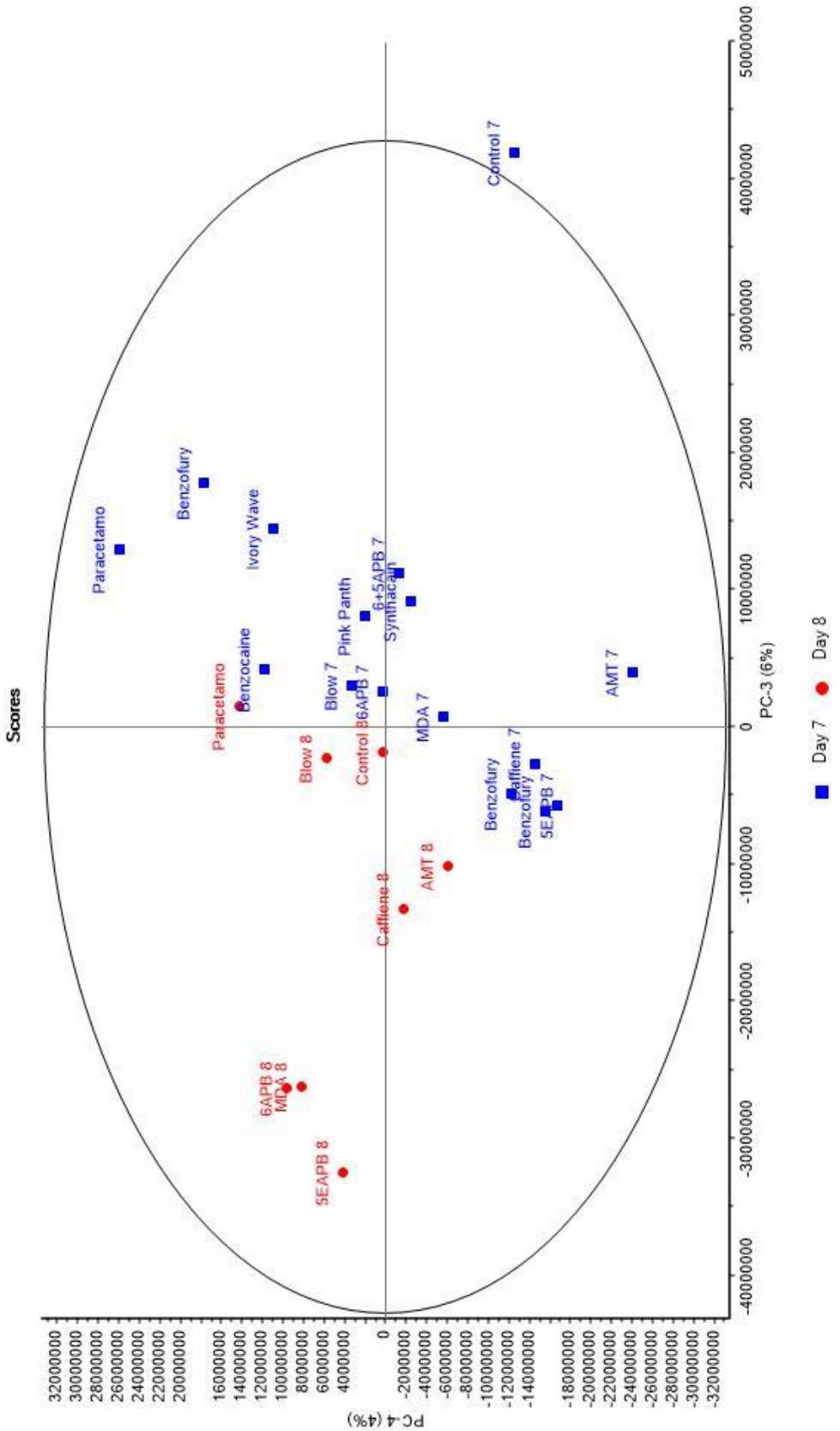


Figure 189: PCA plot of development days 7 and 8 analysed using identified hydrocarbons and peak areas.

6.3.12 Day 9

At sampling day 9 only two populations remained; the control and paracetamol. This is due to early pupariation caused by drug presence in the previously remaining populations. Between these two, 28 hydrocarbons were identified (Table 49). They ranged between 21 and 35 in chain length and 57.1% were mono-methyl alkanes (Table 50). The paracetamol population contained 26 of the hydrocarbons present at this sampling day; the control population samples contained 22 of the identified hydrocarbons.

Table 49: List of all hydrocarbons identified at sampling day 9 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Control	Paracetamol
6-Methylhenicosane	6	12.662	1.39	1.33
Docosene	8	12.815	2.60	2.27
Docosane	9	13.087	9.50	8.84
10-Methyldocosane	11	13.58	2.34	3.58
9-Methyldocosane	12	13.699	1.68	1.83
7-Methyldocosane	13	13.894	0.00	1.79
Tricosene	18	15.559	0.00	3.34
9+11-Methyltricosane	21	16.383	5.81	4.91
7-Methyltricosane	22	16.485	4.89	3.96
Tetracosane	25	17.029	2.98	3.61
10+8-Methyltetracosane	27	17.581	2.39	3.02
Pentacosane	33	19.246	5.62	4.21
9+11-Methylpentacosane	34	19.815	10.24	4.72
Hexacosane	39	20.546	3.14	2.43
Mono-methyl branched hexacosane	41	21.073	2.26	1.99
4-Methylhexacosane	42	21.192	4.21	4.97
11+13-Methylheptacosane	44	21.778	2.48	0.00
Nonacosene	47	22.44	2.60	2.98
Nonacosane	48	22.942	6.70	4.62
11+13-Methylnonacosane	49	23.511	9.22	3.63
7-Methylnonacosane	51	23.672	1.85	0.00
Mono-methyl branched nonacosane	53	24.309	0.00	1.57
Hentriacontene	60	26.017	6.59	7.97
Hentriacontene	61	26.246	0.00	2.53
Hentriacontane	63	26.722	0.00	4.50
9+11-Methylhentriacontane	64	27.283	3.90	0.40
Tritriacontene	69	29.305	2.56	6.37
Tritriacontene	71	29.509	1.42	3.24
7-Methyltritriacontane	74	29.891	3.62	1.78
Pentatriacontene	81	30.825	0.00	3.61

Table 50: A breakdown of the hydrocarbon types analysed at sampling day 9.

Day 9	Percentage
Alkenes	21.4
Alkanes	21.4
Mono-methyl branched Alkanes	57.1

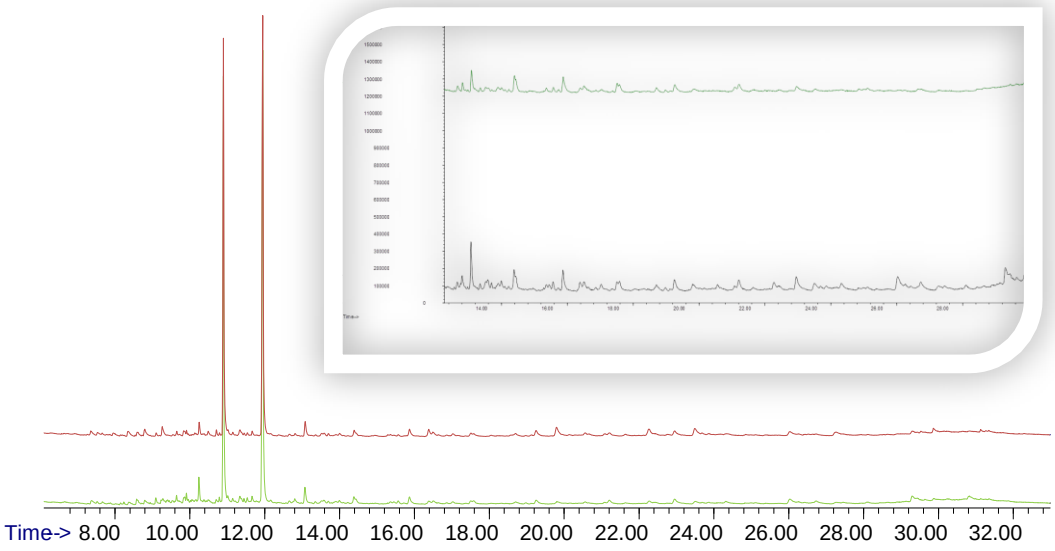


Figure 190: Day 9 control and paracetamol diet chromatograms for comparison.

6.3.13 Day 10

At day 10, only paracetamol samples remained as the control had pupariated. The population showed large numbers of hydrocarbons, 40 were identified (Table 51) and 65% were mono-methyl alkanes and they ranged between 21 and 35 in chain length (Table 52). Comparison cannot be made due to the pupariation of the control population.

Table 51: List of all hydrocarbons identified at sampling day 10 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Paracetamol
9+11-Methylhenicosane	1	12.152	0.55
Mono-methyl branched henicosane	3	12.339	0.41
7-Methylhenicosane	5	12.56	0.30
6-Methylhenicosane	6	12.662	0.64
Docosene	7	12.73	0.88
Docosane	9	13.087	9.12
11-Methyldocosane	10	13.35	0.67
10-Methyldocosane	11	13.58	1.60
9-Methyldocosane	12	13.699	0.94
7-Methyldocosane	13	13.894	1.26
6-Methyldocosane	14	13.996	1.45
5-Methyldocosane	15	14.208	0.86
2-Methyldocosane	17	14.905	0.95
Tricosene	18	15.559	1.38
Tricosane	20	15.857	6.30
9+11-Methyltricosane	21	16.383	2.47
7-Methyltricosane	22	16.485	2.13
Mono-methyl branched tricosane	24	16.85	0.67
Tetracosane	25	17.029	2.18
Tetracosane	26	17.496	1.33
10+8-Methyltetracosane	27	17.581	1.94
Pentacosene	30	18.736	1.37
Pentacosane	33	19.246	6.98
9+11-Methylpentacosane	34	19.815	2.95
9-Methylpentacosane	35	19.943	1.29
Hexacosane	39	20.546	3.49
Mono-methyl branched hexacosane	41	21.073	0.96
4-Methylhexacosane	42	21.192	1.89
Nonacosene	47	22.44	3.30
Nonacosane	48	22.942	5.57
11+13-Methylnonacosane	49	23.511	2.16
7-Methylnonacosane	51	23.672	1.39
Mono-methyl branched nonacosane	53	24.309	2.94
Hentriacontene	60	26.017	4.14
Hentriacontene	61	26.246	2.75
Hentriacontene	62	26.416	3.06
Hentriacontane	63	26.722	3.35
9+11-Methylhentriacontane	64	27.283	0.64
6-Methylhentriacontane	66	27.444	0.93
Tritriacontene	69	29.305	1.86
Tritriacontene	70	29.424	1.95
Tritriacontene	71	29.509	3.45
7-Methyltritriacontane	74	29.891	1.29
6-Methlytetratriacontane	79	30.664	0.70
Pentatriacontene	81	30.825	3.56

Table 52: A breakdown of the hydrocarbon types analysed at sampling day 10.

Day 10	Percentage
Alkenes	17.5
Alkanes	17.5
Mono-methyl branched Alkanes	65.0

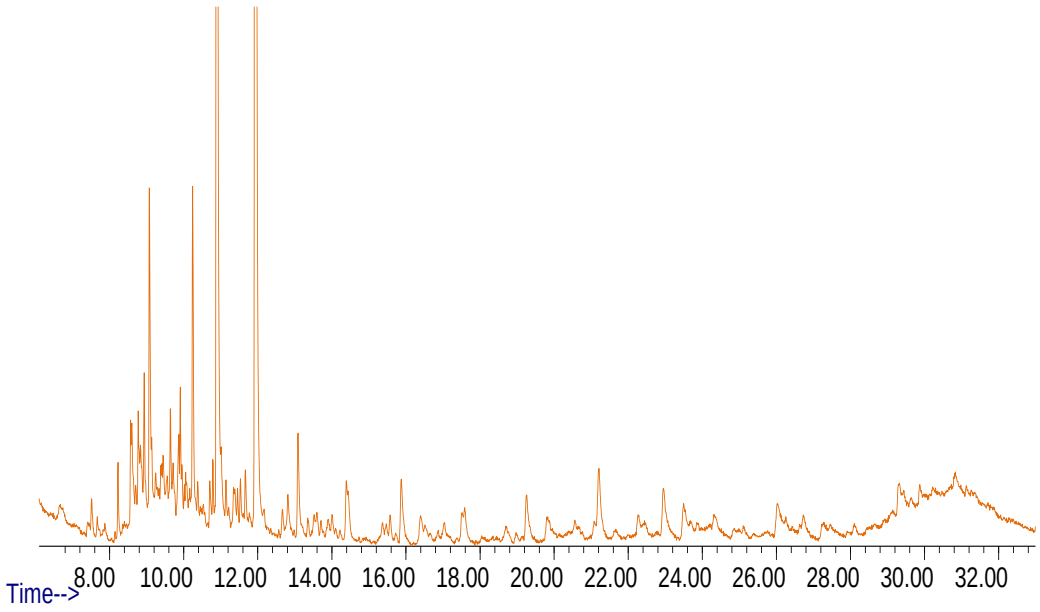


Figure 191: Day 10 paracetamol diet chromatogram.

6.3.14 Day 11

Day 11 continued to show a large number of identifiable hydrocarbons for paracetamol spiked samples. In total, 45 were identified (Table 53) and of those 68.9% were mono-methyl alkanes (Table 54). They ranged between 21 and 35 in chain length. Again, no comparison can be made due to the absence of the control sample.

Table 53: List of all hydrocarbons identified at sampling day 11 (%).

Hydrocarbon Identification	Peak Number	Retention Time	Paracetamol
Mono-methyl branched henicosane	3	12.339	0.58
6-Methylhenicosane	6	12.662	0.97
Docosene	7	12.73	0.86
Docosane	9	13.087	0.64
11-Methyldocosane	10	13.35	0.42
10-Methyldocosane	11	13.58	2.51
9-Methyldocosane	12	13.699	2.14
7-Methyldocosane	13	13.894	1.03
6-Methyldocosane	14	13.996	2.49
2-Methyldocosane	17	14.905	1.52
Tricosene	18	15.559	1.82
Tricosane	20	15.857	5.25
9+11-Methyltricosane	21	16.383	6.53
7-Methyltricosane	22	16.485	3.22
6-Methyltricosane	23	16.63	1.24
Mono-methyl branched tricosane	24	16.85	1.33
Tetracosane	25	17.029	3.24
Tetracosane	26	17.496	1.29
10+8-Methyltetracosane	27	17.581	2.68
Mono-methyl branched tetracosane	28	18.159	0.78
Pentacosene	30	18.736	1.44
Pentacosene	31	18.898	0.97
Pentacosene	32	18.957	0.65
Pentacosane	33	19.246	6.64
9+11-Methylpentacosane	34	19.815	7.73
5-Methylpentacosane	36	20.147	1.19
4-Methylpentacosane	37	20.283	0.61
2-Methylpentacosane	38	20.393	1.22
Hexacosane	39	20.546	3.82
6-Methylhexacosane	40	20.648	2.30
Mono-methyl branched hexacosane	41	21.073	1.54
4-Methylhexacosane	42	21.192	2.16
Heptacosene	43	21.48	1.51
Nonacosene	47	22.44	1.43
Nonacosane	48	22.942	5.88
11+13-Methylnonacosane	49	23.511	3.56
7-Methylnonacosane	51	23.672	1.77
5-Methylnonacosane	52	23.842	1.38
Mono-methyl branched nonacosane	53	24.309	2.47
Mono-methyl branched nonacosane	55	24.989	0.63
Mono-methyl branched nonacosane	56	25.108	0.81
Hentriacontene	60	26.017	1.27
Hentriacontene	61	26.246	1.20
Hentriacontene	62	26.416	0.70
Hentriacontane	63	26.722	1.50
9+11-Methylhentriacontane	64	27.283	1.22
5-Methylhentriacontane	67	28.651	0.44
4-Methylhentriacontane	68	29.007	0.17
Tritriacontene	69	29.305	0.37
Tritriacontene	70	29.424	0.44
Tritriacontene	71	29.509	0.69
Tritriacontene	72	29.627	0.56
7-Methyltritriacontane	74	29.891	0.78
Pentatriacontene	81	30.825	0.37

Table 54: A breakdown of the hydrocarbon types analysed at sampling day 11.

Day 11	Percentage
Alkenes	15.6
Alkanes	15.6
Mono-methyl branched Alkanes	68.9

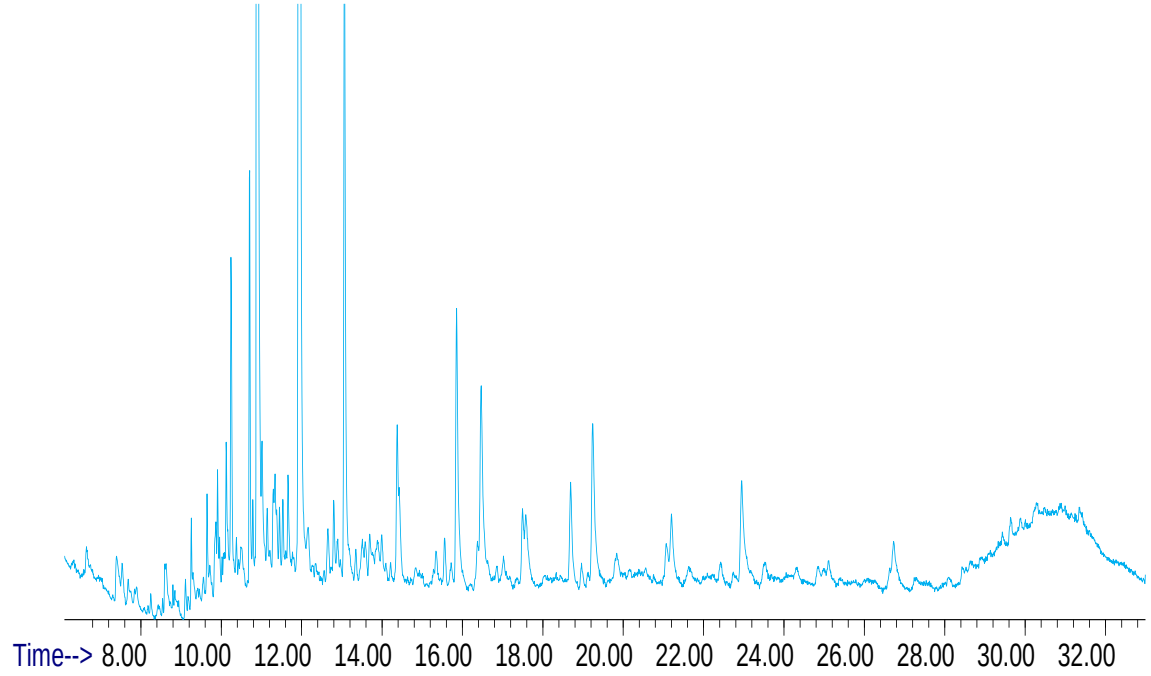


Figure 192: Day 11 paracetamol diet chromatogram.

6.4 Overall Conclusion

Previous research has shown promising results for the analysis of cuticular hydrocarbons from forensically important blowfly [187], [189], [191], [206], [221], [234], [235], [313]–[318]. Focus has remained on larval aging and species identification. Both accurate ageing and species determination are imperative when estimating post mortem interval. Cuticular hydrocarbons showed clear differences between life stages and age when sampling *C.vomitoria*, *C.vicina* and *P.terraenovae*, analysing with GC-MS [319]. The usefulness was further proved in further research focussed on aging and identifying *C.vicina*, *C.vomitoria* and *L.sericata* where analysis again showed great potential [221], [238].

Analysis within this study, focussed on the whole profile was carried out and principle component analysis showed a large amount of scattering, differences were observed but this would not be sufficient to accurately age blowfly larvae. Focussed analysis followed this and an overall pattern was observed relating the sampling days in a clockwise circular pattern, 6 clusters were noted, days 1 and 2 were grouped as were days 7 and 8. Days 9 onwards were harder to determine due to reduced samples following early pupariation. Samples found to group when analysed with the whole population were reanalysed in pairs and separation was shown to be possible. Few differences within the sampling days were observed between populations; the main outlier appeared to be the control population at day 7, which did not cluster with other day 7 samples. This requires further investigation to determine the origin and reasoning behind this variance. The profiles observed appeared to be dominated by methyl branched alkanes, this was also observed during the analysis of surface lipids of weevils [320]. Discrimination between larval age

appears to be as a consequence of mainly cuticular hydrocarbon concentration as opposed to hydrocarbon presence [319]. It appears from the results of this experiment that cuticular hydrocarbons are not affected by the presence of drugs within their food source; a reduction in scattering is seen when compared with analysis of the whole extract prior to integration of specified peaks and this selection increases the accuracy of ageing of blowfly larvae.

Analysis of length and weight developmental data shown in chapter 5 described a post mortem interval estimation error of up to 48 hours, the results of the work presented within this chapter show encouraging results for accurate PMI estimation when drugs are concerned. Larval samples for analysis at crime scenes are monopolised by the third instar. This allows for a larger time frame for larval age, estimation using cuticular hydrocarbon analysis could narrow this estimation and greatly improve accuracy. Cuticular hydrocarbons are known to be very stable.

Previous research has shown strong correlation between the thickness of the waxy cuticle (and therefore concentration of cuticular hydrocarbons) and the relative humidity and average temperatures the larvae are exposed to [314]. Water loss prevention is a known use of cuticular hydrocarbons in insect species [187]. Insects have the ability to regulate the production of cuticular hydrocarbons in response to environmental conditions in order to prevent desiccation [314]. The need for waterproofing of the insect cuticle will increase as the larvae start to wander in search of dry, dark environments for pupariation, the need for this increased hydrocarbon production would also increase with a rise in temperature. It must be considered that it is possible for drugs present within a larval diet, to have the effect of changing the immediate environment, for example; alteration

of feeding rate or behaviour causing a change in temperature, which may then affect hydrocarbon production. There are two factors known to influence the composition of cuticular hydrocarbons, genetic and environmental. It is likely that any changes found within the cuticular hydrocarbon profile in response to drugs would be due to change caused to the larval environmental not a change in development rate.

One study did show a change in cuticular hydrocarbon profile in response to diet [202]. Three diets were tested on an ant species (*Linepithema humile*), two hydrocarbon rich, insect diets and one artificial non-insect diet. Each diet was shown to alter the profile analysed by contributing diet specific cues. This effect has been questioned however, due to the speed of the observed effect [187].

Cuticular hydrocarbons are known to change over time as new compounds are synthesized. Although the results shown within this chapter presents the capacity to estimate larval age within the incorporation of error from drug presence, it must be considered that current studies have been carried out within the lab environment and this does not account for unpredictable factors such as weathering and fluctuating environmental conditions. Further research is necessary to gain a complete understanding of this topic. This would include quantitative analysis of the hydrocarbons present within the samples, as concentration of hydrocarbons appeared to increase as the larval samples age.

7.0 Introduction

7.0.1 Insects as alternative toxicological samples

The use of insects as alternative toxicological samples dates back to the first known publication in 1980 where drugs were detected in larval samples when no other suitable samples remained and so supported a diagnosis of death by intoxication [104]. In skeletonised remains or where the decomposition is too advanced, it has been shown that insects can be a suitable alternative for analysis [126], [105].

Following this study, the area has been heavily researched, for a more detailed overview of this; the reader is directed to chapter 1. Many attempted to investigate the correlation between larval drug concentration and human tissue drug concentration, and it has been suggested that quantification of these compounds is unpredictable and unreliable [119], particularly regarding multiple drugs and differing concentrations.

Numerous factors will affect potential drug concentration such as; species, human tissue type, species instar, initial drug concentration and a number of other variables. The chemical properties of the drugs are shown to greatly affect detection this will also determine metabolism, elimination and accumulation [321].

Despite this, even as only qualitative samples, blowfly larvae can be considered as alternative samples for drug detection, especially in actively feeding larvae [94]. Some studies have shown detection within the larvae but not within the human tissue sample [121], [122], [126] whilst the opposite has also been shown [118].

Detection of drug presence within the larval samples of this study is also of interest to confirm that the substance has been ingested by the larvae. Developmental differences have been shown in chapter 5; it is fair to assume that this is due to drug presence as all other variables remained constant. The detection of the substances within the larvae would however, confirm this.

7.0.2 NPS and adulterants background

Caffeine

Caffeine is a stimulant, psychoactive drug which is legal. The purpose for including it in this study is for two reasons, firstly caffeine is a common adulterant of Novel Psychoactive drugs and is therefore likely to be found during their analysis (see chapter 5 for further information), it was highlighted as a concern due to the high concentrations recovered from such products that an overdose could occur [178]. Caffeine is also the worlds most widely consumed psychoactive drug and is legal and not regulated. Caffeine overdose can result in death [322], [323].

Paracetamol

Paracetamol is considered a mild analgesic, meaning it treats pain. It is an unscheduled drug. This was included in this study due to its popularity as an adulterant in compounds such as NPS. The misuse of analgesic drugs is responsible for many deaths each year; paracetamol was mentioned on the death certificate of 1024 individuals between the years 2012 and 2016 in the UK alone [304].

Benzocaine

Benzocaine is part of this study as it is a common adulterant of illegal drugs; the reader is directed to chapter 5 for more information on this topic. Its use is normally as a local anaesthetic. In the United Kingdom, medication is governed by the Medicines Regulations 2012 where all medication will fall into one of three categories. Benzocaine is listed under General Sales List (GSL) meaning it is available off the shelf with no medical training needed in order to sell it [304].

Benzofury and 6APB

As previously explained, Benzofury was the focus of this research. Three different batches are analysed, one beige, one blue and one green. Benzofury was advertised as a novel psychoactive substance with the active ingredient 6APB. 6APB is a psychoactive compound of the substituted benzofuran, substituted amphetamine and substituted phenethylamine classes.

In June 2013 6-APB and a number of its analogues were classified under a temporary class of drugs following recommendations from the Advisory Council on the Misuse of Drugs (ACMD). Later in 2013 it was recommended that these substances should become Class B, Schedule 1 substances. In 2014 it was announced by the UK Home Office that this would go ahead, 6APB and all structurally related drugs would be classified as class B drugs [324], [325], [326]. 6APB is the advertised active compound of the 'Benzofury' substances and can also be purchased separately. 5APB was initially commonplace in online headshops, this was then replaced by 6APB, and apparently displaying the same effects, a supposed mixture of the two was purchased for analysis.

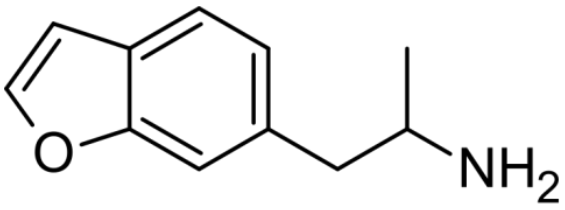


Figure 193: Chemical structure of 6APB.

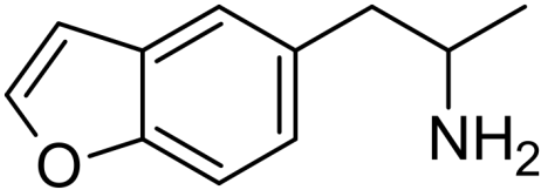


Figure 194: Chemical structure of 5APB

5EAPB

As previously described, new NPS were constantly being synthesized to stay ahead of drug control. 5EAPB was one such drug, synthesised and sold as a 6APB or 5APB alternative. The difference in chemical is shown in Figure 195 as compared with Figure 193 and Figure 194.

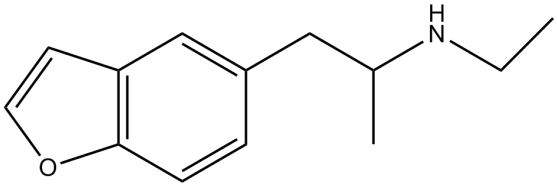


Figure 195: Chemical structure of 5EAPB.

AMT

AMT was shown to be a popular and widely used drug when initially beginning this research project, see chapter 1 for further details. AMT is a stimulant of the tryptamine class. It is a class A drug in the UK.

Synthacaine

Synthacaine is a NPS which is supposed to provide an alternative to cocaine, an illegal class A substance. The contents are unknown and not specified although drug forums have discussed the possibility of it containing Dimethocaine, a local anaesthetic with stimulant properties shown to be half the potency of cocaine.

Pink Panther

'Pink panther' is another unknown NPS. Although the content is unknown, drug forums have hypothesized that it includes Methiopropamine (MPA) or 2-Aminoindane (2AI). MPA is structural analogue of methamphetamine; 2AI is a pre-analogue of amphetamine.

Ivory Wave

Ivory wave is an unknown NPS. Drug forums suspect the compound to contain Desoxypipradrol which is structurally related to Pipradrol, a mild central nervous system stimulant.

Blow

Blow is an unknown NPS. Drug forums have hypothesized that it includes Methiopropamine (MPA). MPA is structural analogue of methamphetamine.

MDA

MDA (3, 4-Methylenedioxyamphetamine) is a recreational drug within the amphetamine family. MDA was considered the comparison drug to 6APB. The decision was therefor made to include the analysis of MDA for comparison with 6APB.

7.0.3 Drug analysis of Novel Psychoactive Substances

Novel psychoactive substances (NPS) have been around for a long period of time, appearing first in 1772 with the discovery of nitrous oxide. These substances became prominent in more recent years with the government introducing the psychoactive substances act in 2016. Prevalence of these novel drugs increased research focussed on the composition and effects, particularly in post mortem samples. The reader is directed to chapter 1 for further information regarding this topic.

7.0.3.1 Presumptive drug testing

Presumptive tests are used to give an indication of the type of substance present, in this case the drug group. This would then be followed by a confirmatory technique, in this thesis the identity of the substance is confirmed with GC-MS.

Presumptive tests often take the form of colour testing where the unknown substance is mixed with selected chemicals and where a colour is produced when specific things are present. Different tests can be used to define different drug groups. Six presumptive tests were selected for analysis of the novel psychoactive substances presented within this thesis, Mandelin reagent, Mecke reagent, Marquis reagent, Dille-Koppanyi reagent, Robodope reagent and Ferric chloride reagent. Mandelin is used as a simple test to identify alkaloids, mainly naturally occurring chemical compounds, along with other things. Mecke and Marquis are also used for this purpose and all three can be used to determine amphetamines and their derivatives. Ferric chloride is used to determine the presence of phenol groups. Robodope reagent is mainly used due to its reaction with primary amines enabling distinction of MDA from MDMA type substances. Finally, Dille-

Koppanyi reagent is mainly used to presumptively identify barbiturates. All six presumptive tests will be carried out on all novel psychoactive substances, due to their unpredictable nature. A table showing possible spot test result meanings is shown in the appendix (Figure 275).

7.0.3.2GC-MS

This is the combination of two techniques to form a singular method to analyse a mixture of compounds, including drugs qualitatively or quantitatively. The GC will separate the components and the MS will characterise each individually. GC-MS has been used since the 1960s [187] and has become the tool of choice, it provides powerful separation, identification and is user friendly [262]. GC-MS is a confirmatory technique.

7.1 Aims and objectives

The focus of this thesis has been on NPS. These substances are known to be unpredictable in their composition. The analysis of the drugs included within this study is of interest for interpretation of the results produced. As previously explained, the use of insects as alternative toxicological samples is heavily researched and of interest in relation to all compounds of forensic importance.

This chapter focusses firstly on analysis of compounds selected within this thesis, by means of presumptive testing and GC-MS; this is followed by examination and interpretation of samples taken from larvae feeding on these drugs, focused upon analysing the internal composition to determine if the drugs are present in their non-metabolized form.

7.2 Materials and methods

Novel Psychoactive Substances of interest were originally selected according to popularity at the time of initiating this research, as shown in chapter 1. APB (aminopropylbenzofuran) compounds were the focus; 6APB and 5APB were popular and widely used. These compounds came in a pure form, 6APB, 5APB and a mix of the two 6 and 5APB, a popular NPS named Benzofury was sold based on its active compound being 6APB. Many batches of this specific NPS existed and three are tested here. As previously explained, new NPS were constantly being synthesized to stay ahead of drug control. 5EAPB was one such drug, synthesised and sold as a 6APB or 5APB alternative. AMT was also highlighted in chapter 1 as a popular drug with widespread use. During initial stages of research, a number of other drugs were acquired after enquiring with online headshops to determine their best-selling drugs, through this method Blow, Pink Panthers, Synthacaine and Ivory Wave were purchased.

7.2.1 Presumptive testing of novel psychoactive drugs

Chemicals used for production of presumptive test reagents are listed in chapter 3. Prior to testing with the reagents, powders or tablets were observed, these observations were noted. Tablets were crushed. A few milligrams of each drug were added to a testing well, where the prepared reagents were then added using a dropper bottle. Initial reactions were observed, these comments were noted up to 15 minutes to ensure a complete reaction. Each of the 11 NPS and 3 adulterants were tested with all six reagents listed.

Mandelin reagent

The Mandelin reagent was composed of ammonium vanadate in solution and concentrated sulphuric acid, 1.25 g was weighed and added to 3.75 ml of distilled water and then 246.25 ml of concentrated sulphuric acid. Once mixed, the solution was added to a dropper bottle and 5 drops of the reagent was added to each compound in each well.

Mecke reagent

The Mecke reagent was composed of 2.5 g of Selenious acid added to 250 ml of concentrated sulphuric acid. This was again added to a dropper bottle for ease and 5 drops added to each compound in each well.

Marquis reagent

The Marquis reagent was composed of 225 ml of concentrated sulphuric acid added to 25 ml of 40% formaldehyde, mixed and then added to a dropper bottle and 5 drops added to each compound in each well.

Dille-Koppanyi reagent

The Dille-koppanyi reagent has 2 solutions, solution A is produced by adding 0.25 g of cobalt (II) acetate tetrahydrate to methanol (250 ml) and glacial acetic acid (0.5 ml). Solution B is produced by adding 12.5 ml of isopropyl amine to 237.5 ml of methanol. Both solutions are kept in separate dropper bottles. 4 drops of each solution are added to each drug in succession.

Robodope reagent

Robodope reagent is composed of two solutions. Solution A was produced by adding 1 g of sodium nitroprusside to 50 ml of distilled water, then 2ml of Acetone. Solution B was produced by adding 2 g of sodium carbonate to 100 ml of distilled water. Both were added to separate dropper bottles. 1 drop of solution A and 2 drops of solution B was added to each substance in each well.

Ferric chloride reagent

Ferric chloride reagent was produced by adding 8.25 g of ferric chloride (hexa-hydrate) to 250 ml of distilled water, thoroughly mixing and added 5 drops to each drug tested.

7.2.2 GC-MS Analysis of novel psychoactive drugs

Solutions of the NPS are firstly made up in ethyl acetate at a concentration of 1 mg/ml. A sample was then prepared using the initial solution and diluting it 1:1. This was used for analysis on the GC-MS. It was concluded from previous experimental work shown in chapter 4 that either Ethyl acetate or Dichloromethane can be utilised with no detrimental effect due to insolubility. Solutions for analysis with GC-MS need to be derivatized, each drug sample was analysed both with and without derivatisation. HFBA performed overall the best, across all analysed compounds. Given the unpredictable nature of NPS, this is the obvious choice for use as a derivatising agent.

GC-MS Program

Data from the GC-MS was processed using Agilent Chemstation software. The oven temperature program was as follows: hold at 100 ˚C for 3 minutes then ramp to 140 ˚C at

20 ˚C/min and hold for 2 minutes. Ramp again to 150 ˚C at 5 ˚C/min and hold for 2 minutes. Ramp to 160 ˚C at 5 ˚C/min and hold for 5 minutes. Finally, ramp to 250 ˚C at 20 ˚C/min. Elution was carried out at 1mL/min with helium. This program was chosen in order to separate peaks eluting closely together.

7.2.3 GC-MS Analysis of internal compounds

Samples previously frozen in Eppendorf tubes during sampling were thawed and then homogenised using narrow scissors. 1 mL of 50:50, methanol: DCM was added to 500 mg of sample, along with Zirconia/Silica beads to encourage the breakdown of larval tissues. 50mg of magnesium sulphate was also added at this stage to remove water which was causing the solvent mixture to form two layers. The sample was then vortexed and sonicated in an ultra-sonic bath for 15 minutes. The solvent mixture was chosen due to experimentation with sample drug solubility. At this stage the sample was centrifuged to encourage separation, liquid resulting from this was then separated for analysis. The resulting liquid was dried down under a stream of nitrogen, derivatized and then the solvent was added, transferred to an insert for analysis using the auto sampler connected to the GC-MS. The temperature program is as previously described for drug analysis.

7.2.3.1 Testing of analysis method

To ensure that the presence of drugs would be detected if they were present within the larval samples, the analysis method was tested. A selection of larval samples were spiked with a low dosage of an NPS. The usual procedure was then undertaken involving solvent extraction and derivatisation, drying down of the sample and finally reconstituting for analysis.

7.2.3.2 Derivatisation method

For derivatisation of the samples using HFBA, the following protocol was followed. The samples were dissolved in 50 µl of ethyl acetate, 50 µl of the reagent was then added to the sample. The samples were then heated to 70°C for 30 minutes and then allowed to cool to room temperature before placing under a stream of nitrogen to dry completely. 50 µl of ethyl acetate was then added to the sample and the sample was transferred to an insert before analysis with GCMS.

7.3 Results and Discussion

7.3.1 Drug analysis

Drug analysis is presented in two parts, firstly the presumptive testing using six different reagents and secondly the confirmatory analysis with GC-MS. For analysis of many of the NPS included, non-derivatized samples were used due to reference spectra availability.

7.3.1.1 Caffeine

Caffeine was sourced from Acros Organics and is in the form of a solid white/yellow powder. Presumptive test results for caffeine are shown in Table 55. No change was expected with all presumptive tests. Mandelin and Ferric chloride reagents appear to have turned yellow, this is an unexpected result. It is hypothesized that this is due to a pigment present in the drug as opposed to a colour change. If the caffeine is contaminated or impure then this will be confirmed using GC-MS analysis.

Table 55: Presumptive test results for Caffeine.

Reagent	Colour produced	Notes
Mandelin reagent		Turned the white powder, bright yellow.
Mecke reagent		No reaction observed.
Marquis reagent		No reaction observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		No reaction observed.
Ferric chloride reagent		Turned the white powder, yellow.

Analysis using GC-MS confirmed only one peak was visible (Figure 196). This peak was confirmed as Caffeine using both the National Institute of Standards and Technology (NIST) library, where a 98% match with standard caffeine was shown and the use of characteristic ion fragments (Figure 197), the molecular ion m/z=194 is visible. All NIST library confirmations are included in the appendix.

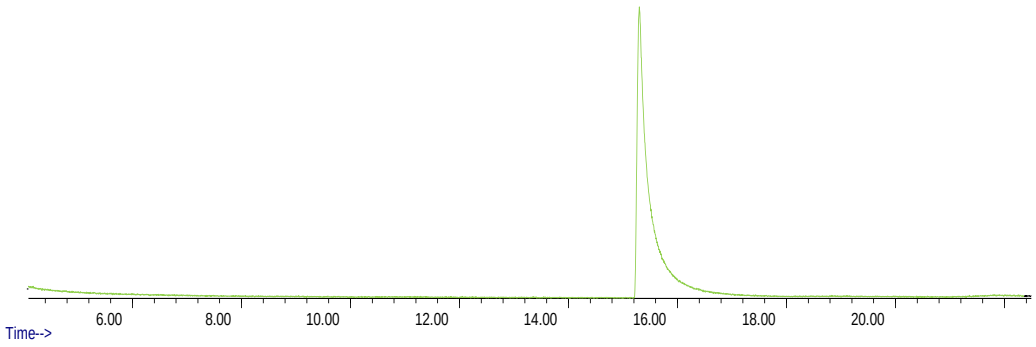


Figure 196: Chromatogram of Caffeine.

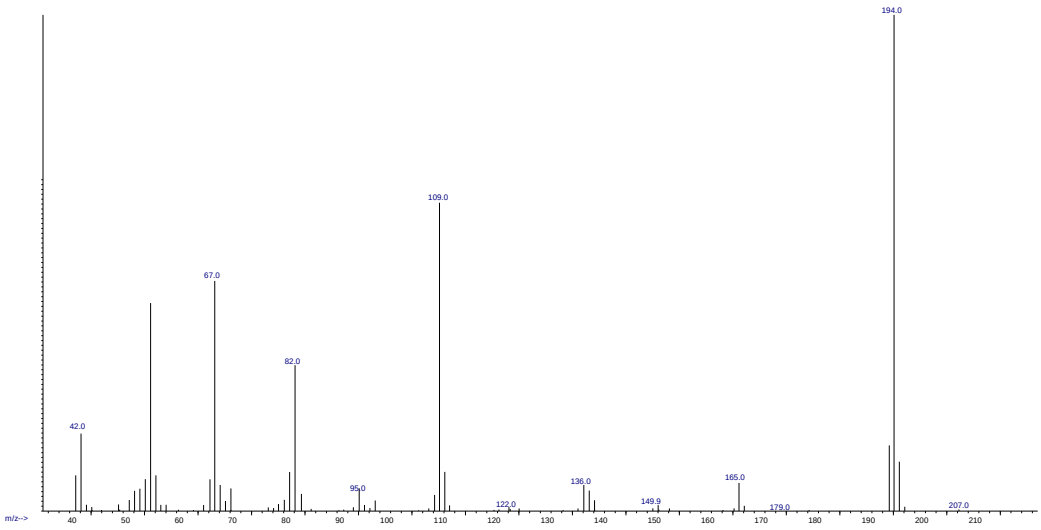


Figure 197: Mass spectrum of ion fragmentation shown by caffeine.

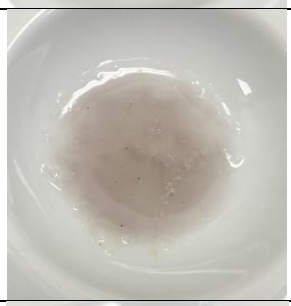
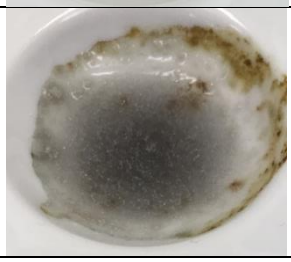
Conclusion

Substance confirmed as caffeine.

7.3.1.2 Paracetamol

Paracetamol tablets were obtained from a local supermarket, it is also known as acetaminophen. It is white in colour and was observed as a powder. Presumptive test results for Paracetamol are shown in Table 56. The colour observed when testing with Ferric chloride is expected in the presence of Paracetamol, the intensity of the colour helps determine the concentration [304]. An olive green is expected with addition of the Mandelin reagent, as observed [304]. No further reactions are expected, and it is suggested that other colour changes observed are due to the pigment contained within the drug. This drug was purchased from a trusted source and therefore contaminants not expected. This was confirmed with GC-MS analysis.

Table 56: Presumptive test results for Paracetamol.

Reagent	Colour produced	Notes
Mandelin reagent		Turned green before changing into a cloudy green colour.
Mecke reagent		Turned pale yellow before changing to a peachy colour in the centre.
Marquis reagent		No reaction immediately, appeared to turn creamy in colour after 5 minutes.
Dille-Koppanyi reagent		Fizzed with the addition of solution A and then turned pale blue in colour.
Robodope reagent		No reaction observed.
Ferric chloride reagent		Changed from yellow to grey with a green/brown rim around the edge.

Analysis using GC-MS confirmed only one peak was visible (Figure 198). This peak was confirmed as paracetamol using the NIST library where a match of 97% with acetaminophen was shown. The fragment ions were also used to confirm this, the molecular ion m/z=151 is shown in the mass spectrum in Figure 199. The peak shown is overloaded due to high concentration.

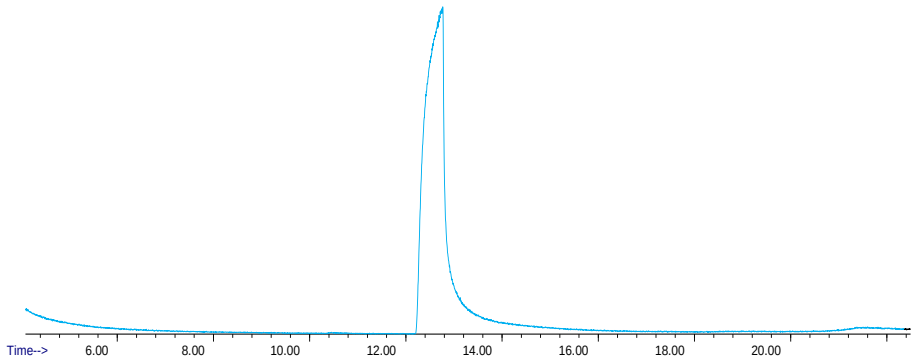


Figure 198: Chromatogram of Paracetamol.

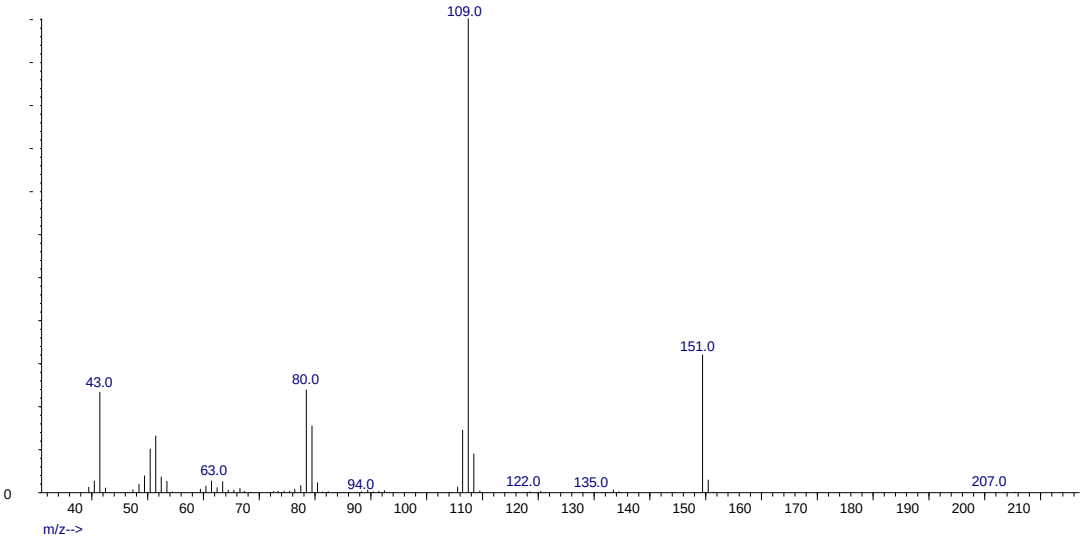


Figure 199: Mass spectrum of ion fragmentation shown by paracetamol.

Conclusion

Substance was confirmed as paracetamol.

7.3.1.3 Benzocaine

Benzocaine was obtained in a large quantity from eBay, it is suggested that it is sold in this manner for bulking of illicit substances. Benzocaine was received in a plastic bag with no other packaging or labelling. It was a white crystalline powder. Presumptive test results are shown in Table 57. Most tests showed no reaction with the substance, this means so far analysis does not disagree with the identity as Benzocaine. Mandelin reagent turned a red/brown colour which is an unexpected result. This colour can be indicative of cocaine along with a number of other drugs such as ketamine. Further analysis is necessary with GC-MS.

Table 57: Presumptive test results for Benzocaine.

Reagent	Colour produced	Notes
Mandelin reagent		Turns a brown/orange colour and then proceeds to turn red/brown.
Mecke reagent		No reaction observed.
Marquis reagent		No reaction observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		No reaction observed.
Ferric chloride reagent		No reaction observed.

Analysis using GC-MS confirmed only one peak was visible (Figure 200). This peak was confirmed as Benzocaine using the NIST library where a match of 96% with a Benzocaine standard was shown. The fragment ions were also used to confirm this, the molecular ion m/z=165 is shown in the mass spectrum in Figure 201.

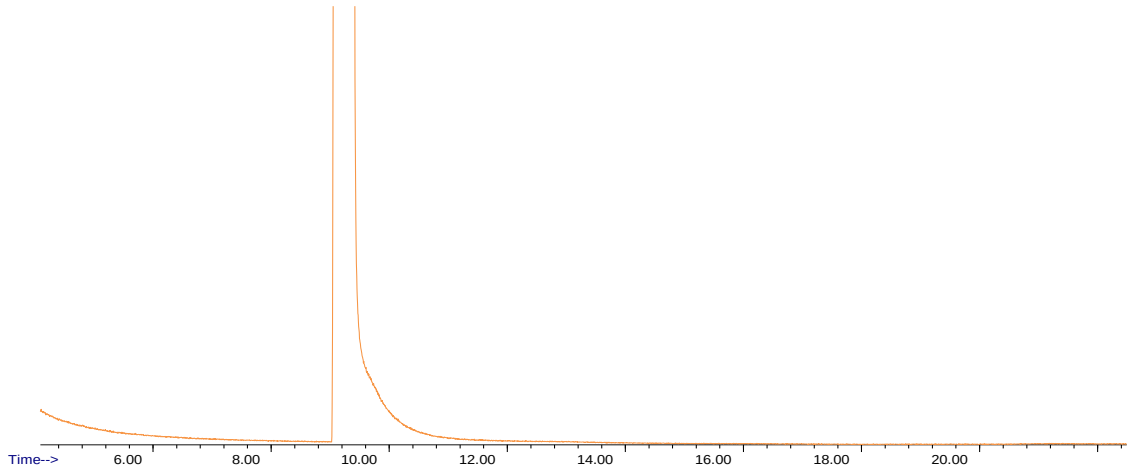


Figure 200: Chromatogram of Benzocaine.

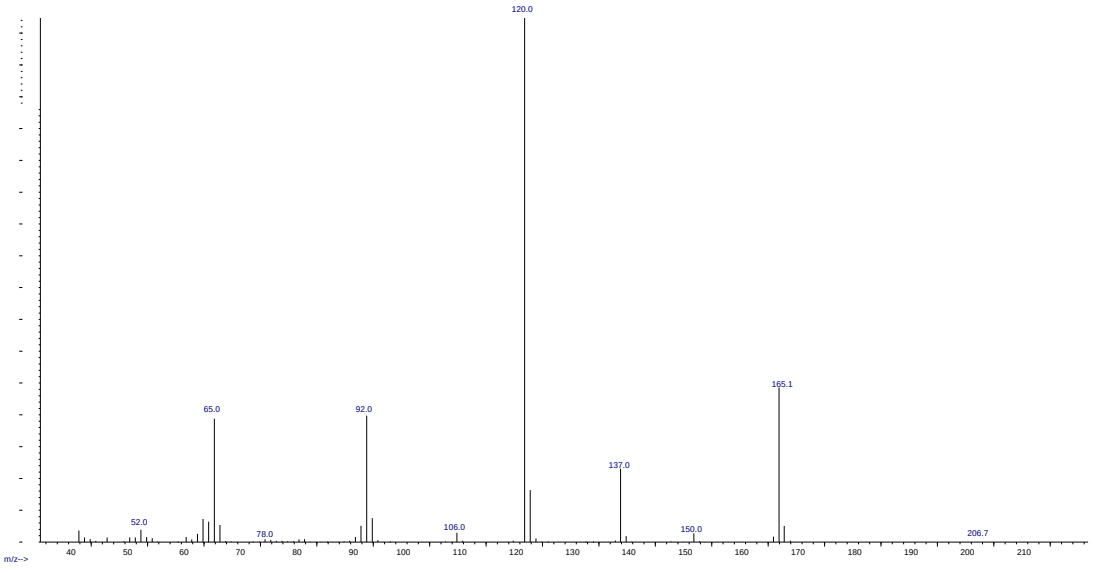


Figure 201: Mass spectrum of ion fragmentation shown by Benzocaine.

Conclusion

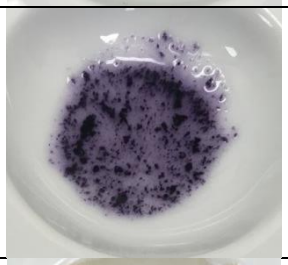
Substance confirmed as Benzocaine.

7.3.1.4 Benzofury Beige

This substance Benzofury (Beige) was purchased from OfficialBenzofury.com. The packaging is shown in chapter three. A beige tablet was included inside the packaging, it was not well formed, and no inscription or symbols were observed.

Presumptive testing results are shown in Table 58. If the active compound is 6APB then a purple colour would be expected for Mandelin, Mecke and Marquis reagents. This is observed during analysis of Benzofury (beige). No reaction is shown for the three other presumptive tests. This indicates that the active compound is 6APB but will be confirmed with GC-MS analysis.

Table 58 : Presumptive test results for Benzofury Beige.

Reagent	Colour produced	Notes
Mandelin reagent		Turned deep purple in colour.
Mecke reagent		Turned purple in colour, with speckles.
Marquis reagent		Purple in colour with darker speckles observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		No reaction observed.
Ferric chloride reagent		No reaction observed.

Analysis using GC-MS confirmed two peaks were visible (Figure 202). Both peaks were confirmed as 6APB or 5APB using the fragment ions, the molecular ion m/z=175 is shown in the mass spectrum in Figure 203. Fragment ions characteristic of 6APB are also shown; ’44, 77, 102 and 131’. Reference spectrum is shown in the appendix.

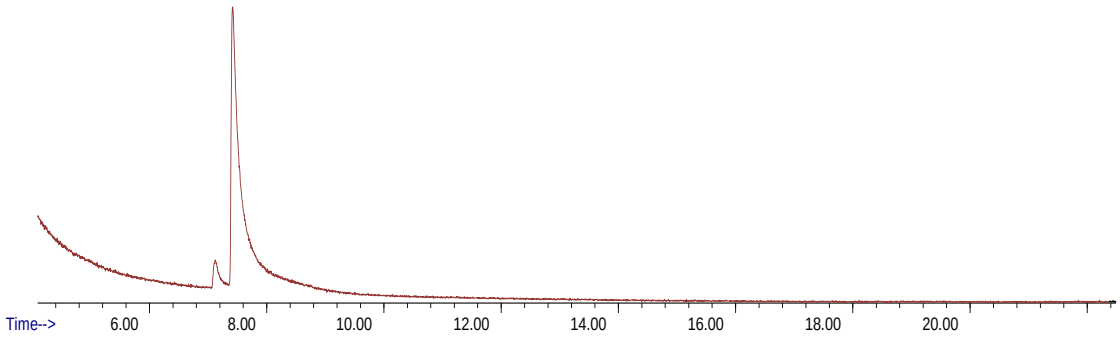


Figure 202: Chromatogram of Benzofury Beige.

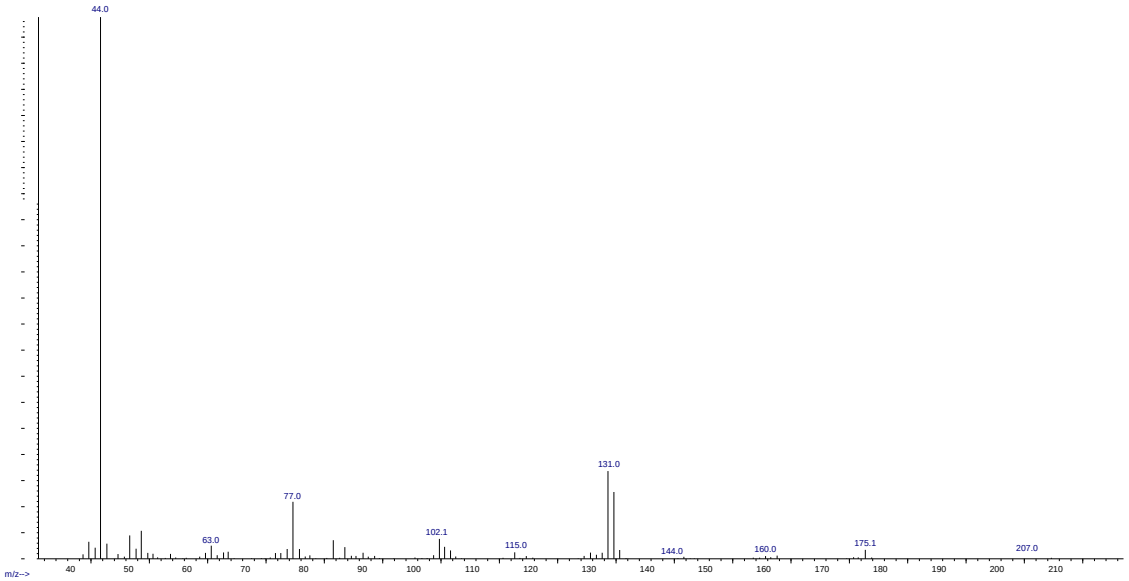


Figure 203: Mass spectrum of ion fragmentation shown by Benzofury Beige.

Conclusion

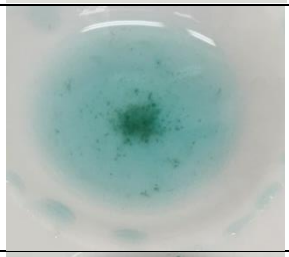
Substance confirmed as 6APB or 5APB, explanation shown in section 7.3.1.10.

7.3.1.5 Benzofury Green

Benzofury (Green) is a separate batch of Benzofury purchased from ViP-Legals.com. The tablet contained was mint green in colour and reasonably well pressed with no inscription or symbols imprinted.

Presumptive testing results are shown in Table 59. If the active compound is 6APB then a purple colour would be expected for Mandelin, Mecke and Marquis reagents. This is observed during analysis of Benzofury (green). No reaction is shown for the three other presumptive tests. This indicates that the active compound is 6APB but will be confirmed with GC-MS analysis.

Table 59: Presumptive test results for Benzofury Green.

Reagent	Colour produced	Notes
Mandelin reagent		Turns a dark purple colour.
Mecke reagent		Turns a grey/purple colour with speckles.
Marquis reagent		Turns a pale purple colour with dark speckles.
Dille-Koppanyi reagent		No reaction observed, colour is due to pigment from the tablet.
Robodope reagent		No reaction observed, colour is due to pigment from the tablet.
Ferric chloride reagent		No reaction observed, colour is due to pigment from the tablet.

Analysis using GC-MS confirmed two peaks were visible, one small and one larger (Figure 204). Both peaks were confirmed as 6APB using the fragment ions, the molecular ion m/z=175 is shown in the mass spectrum in Figure 205 . Fragment ions characteristic of 6APB are also shown; ’44, 77, 102 and 131’. It is apparent that 6APB is lower in abundance here in comparison with Benzofury (Beige).

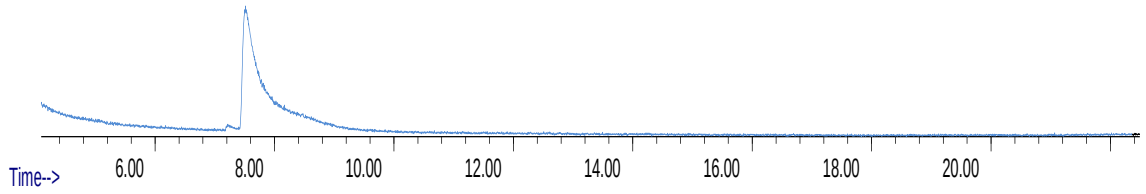


Figure 204: Chromatogram of Benzofury Green.

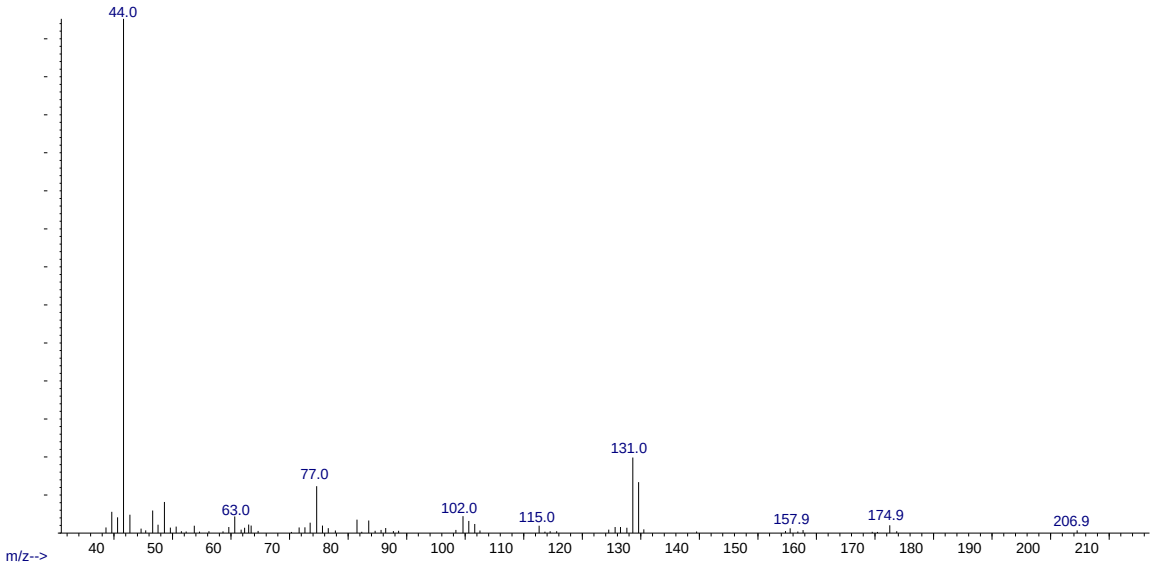


Figure 205 :Mass spectrum of ion fragmentation shown by Benzofury Green.

Conclusion

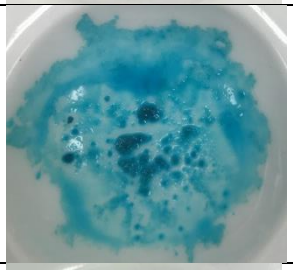
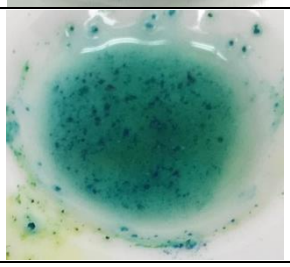
Substance confirmed as 6APB or 5APB, explanation shown in section 7.3.1.10.

7.3.1.6 Benzofury Blue

Benzofury (Blue) is from a separate batch of Benzofury purchased from OfficialBenzofury.com. The tablet contained was aqua marine in colour and well pressed with no inscription or symbols imprinted.

Presumptive testing results are shown in Table 60. If the active compound is 6APB then a purple colour would be expected for Mandelin, Mecke and Marquis reagents. This is observed during analysis of Benzofury (blue). No reaction is shown for the three other presumptive tests; the colour observed is due to the pigmentation of the tablet. This indicates that the active compound is 6APB but will be confirmed with GC-MS analysis.

Table 60: Presumptive test results for Benzofury Blue.

Reagent	Colour produced	Notes
Mandelin reagent		Turns a dark purple colour.
Mecke reagent		Turns a dark purple/blue/black colour.
Marquis reagent		Purple colouring with dark speckles.
Dille-Koppanyi reagent		No reaction observed, colour is due to pigment from the tablet.
Robodope reagent		No reaction observed, colour is due to pigment from the tablet.
Ferric chloride reagent		No reaction observed, colour is due to pigment from the tablet.

Analysis using GC-MS confirmed two peaks were visible, one small and one larger (Figure 206). Both peaks were confirmed as 6APB using the fragment ions, the molecular ion m/z=175 is shown in the mass spectrum in Figure 207. Fragment ions characteristic of 6APB are also shown; ’44, 77, 102 and 131’.

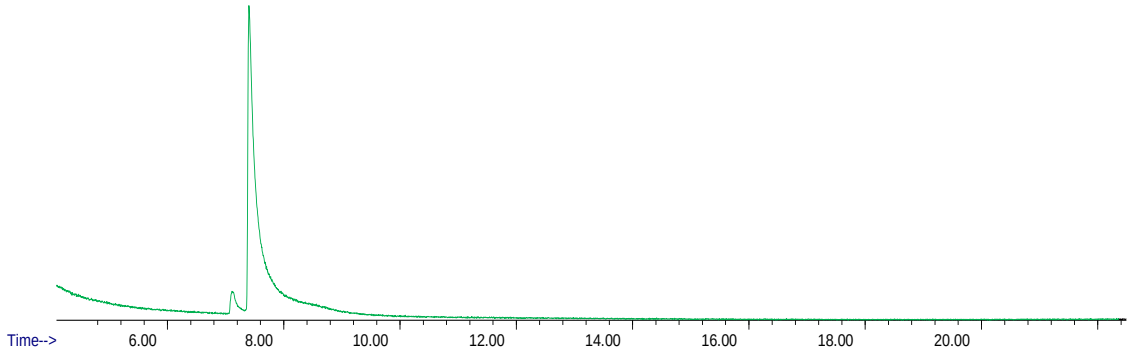


Figure 206: Chromatogram of Benzofury Blue.

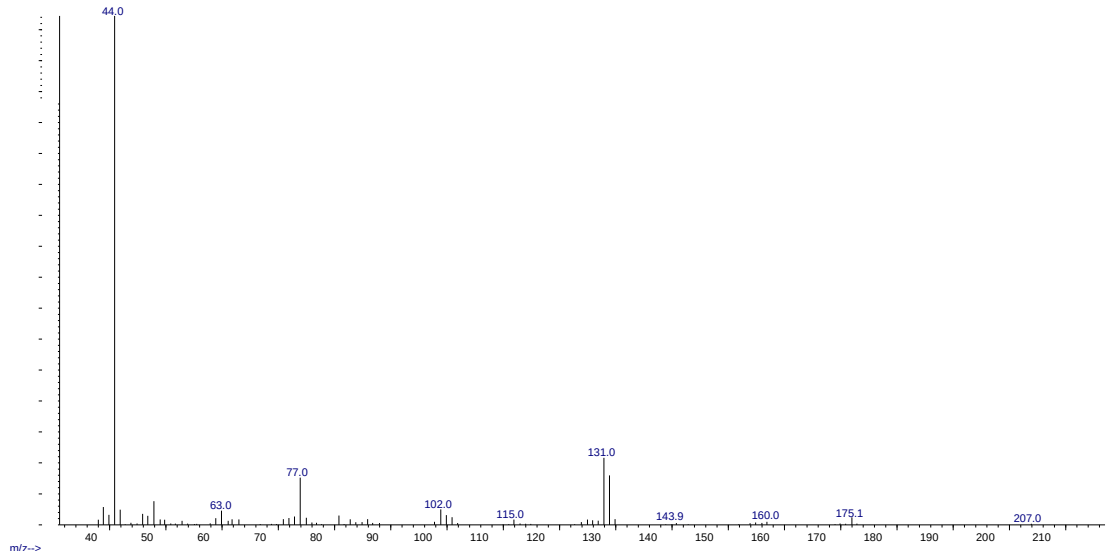


Figure 207: Mass spectrum of ion fragmentation shown by Benzofury Blue.

Conclusion

Substance confirmed as 6APB or 5APB, explanation shown in section 7.3.1.10.

7.3.1.7 6APB

This drug was purchased as '6APB' from ViP-Legals.com. The tablet contained was beige in colour and well pressed with no inscription or symbols imprinted.

Presumptive testing results are shown in Table 61 . If the compound is 6APB then a purple colour would be expected for Mandelin, Mecke and Marquis reagents. This is observed during analysis of Benzofury. No reaction is shown for the three other presumptive tests; the slight colouration observed is due to the pigmentation of the tablet. This indicates that the active compound is 6APB but will be confirmed with GC-MS analysis.

Table 61: Presumptive test results for 6APB.

Reagent	Colour produced	Notes
Mandelin reagent		Colour changes to dark purple/black.
Mecke reagent		Dark purple colour shown with darker speckles, turning darker as time commenced.
Marquis reagent		Violet colouring with dark speckles.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		No reaction observed.
Ferric chloride reagent		No reaction observed, colour is due to pigment from the tablet.

Analysis using GC-MS confirmed four peaks were visible, two small and two larger (Figure 208). Fragment ions were observed, the molecular ion for 6APB is m/z=175 and this is not present in Figure 209. Fragment ions characteristic of 6APB are shown; ’44, 77, 102 and 131’, however ‘56’ is also present.

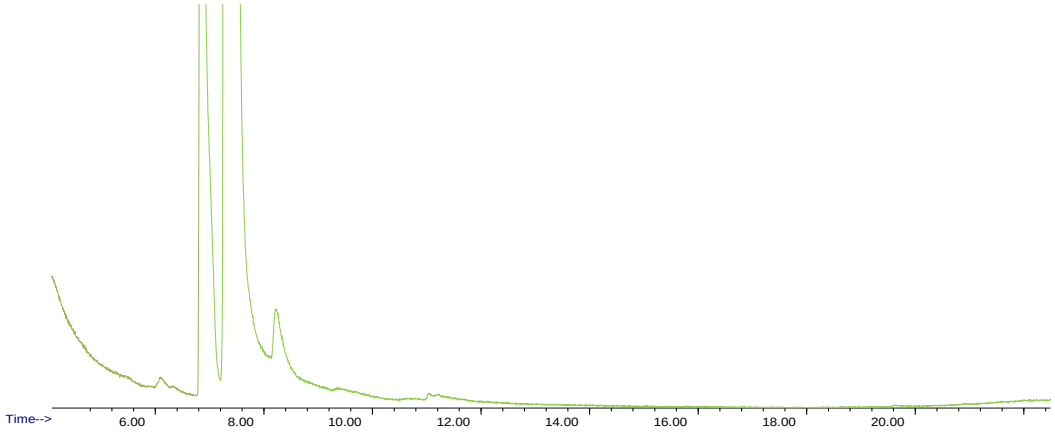


Figure 208: Chromatogram of 6APB.

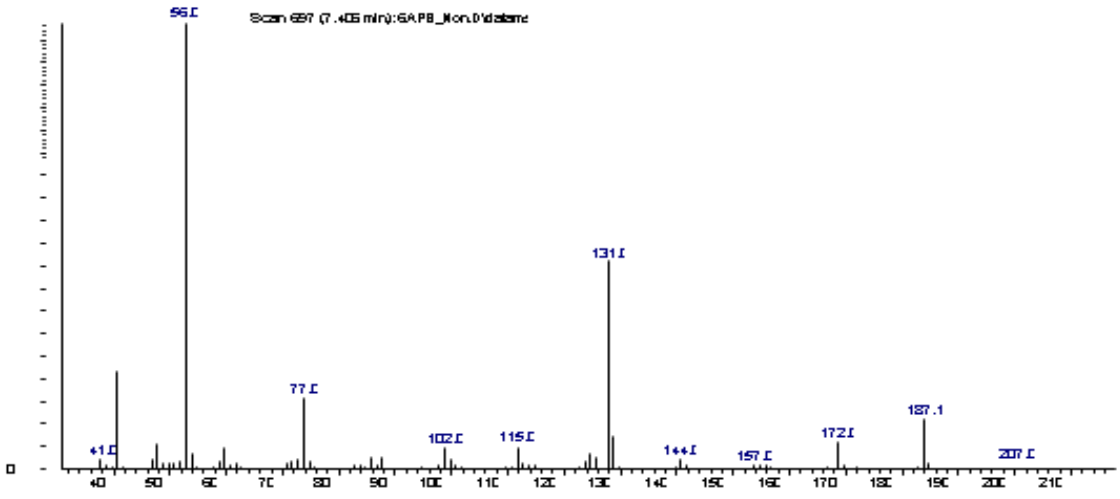


Figure 209: Mass spectrum of ion fragmentation shown by 6APB.

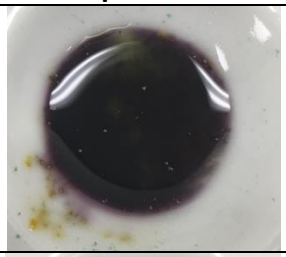
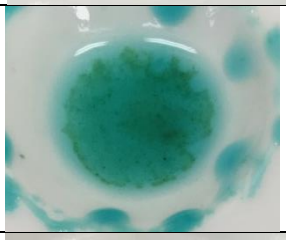
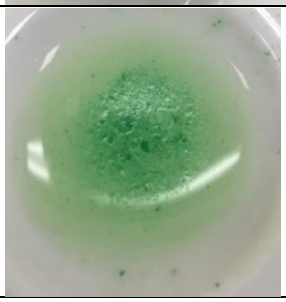
Conclusion

Substance cannot be confirmed as 6APB, it is apparent that a substance very similar in composition is present, but this drug is impure, explanation shown in section 7.3.1.10.

7.3.1.8 6+5APB

This drug was purchased as '6+5APB' from OfficialBenzofury.com. The packaging is shown in chapter 3. The tablet contained was mint green in colour and very well pressed with no inscription or symbols imprinted. Presumptive testing results are shown in Table 62. If the compound contained is 6APB or 5APB or a mix of the two, then a purple colour would be expected for Mandelin, Mecke and Marquis reagents, as before. This is observed during analysis of 6+5APB. No reaction is shown for the three other presumptive tests; the slight colouration observed is due to the pigmentation of the tablet. This indicates that the active compound is either 6APB, 5APB or a mixture of the two; they are extremely similar in composition so although fragment ions can be confirmed with GC-MS analysis, the difference between the two cannot be identified, similarities are shown in Figure 193 and Figure 194.

Table 62: Presumptive test results for 6+5APB.

Reagent	Colour produced	Notes
Mandelin reagent		Turns a dark purple colour.
Mecke reagent		Turns a dark purple/ black colour.
Marquis reagent		Turns a pale violet colour with dark speckles.
Dille-Koppanyi reagent		No reaction observed, colour is due to pigment from the tablet.
Robodope reagent		No reaction observed, colour is due to pigment from the tablet.
Ferric chloride reagent		No reaction observed, colour is due to pigment from the tablet.

Analysis using GC-MS confirmed two peaks were visible, one small and one larger (Figure 210). Both peaks were confirmed as 6APB and/ or 5APB using the fragment ions, the molecular ion m/z=175 is shown in the mass spectrum in Figure 211. Fragment ions characteristic of 6APB and 5APB are also shown; ’44, 77, 102 and 131’.

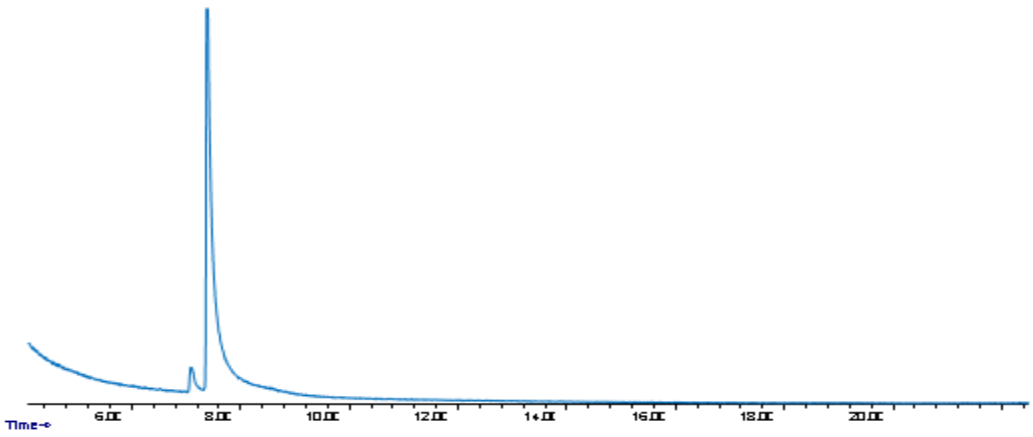


Figure 210: Chromatogram of 6+5APB.

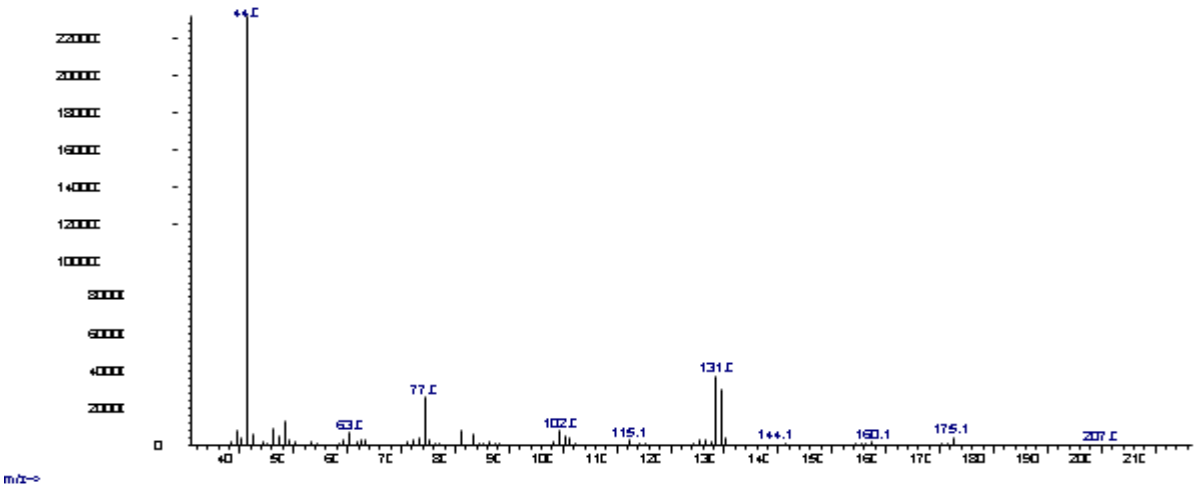


Figure 211: Mass spectrum of ion fragmentation shown by 6+5APB.

Conclusion

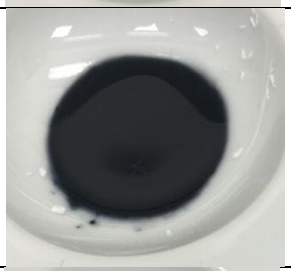
Substance confirmed as 6APB and/ or 5APB, two peaks are present, this could possibly be one for each isomer. An explanation is shown in section 7.3.1.10.

7.3.1.9 5EAPB

5EAPB was purchased from OfficialBenzofury.com. Packaging is shown in chapter 3, the drug was received as a white powder.

Presumptive testing results are shown in Table 63. If the compound is 5EAPB then a purple colour would be expected for Mandelin, Mecke and Marquis reagents as shown before with other APB compounds. This is observed during analysis of 5EAPB. No reaction is shown for the three other presumptive tests; the slight colouration observed is due to the pigmentation of the tablet. This indicates that the active compound is an APB derivative but will be confirmed with GC-MS analysis.

Table 63: Presumptive test results for 5EAPB.

Reagent	Colour produced	Notes
Mandelin reagent		Turned dark purple, almost black, after 10 minutes, a yellow rim appeared to the spot.
Mecke reagent		Fizzing was observed, dark purple colour appeared and then turned even darker almost black.
Marquis reagent		Dark purple/ black colour observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		No reaction observed.
Ferric chloride reagent		No reaction observed.

Analysis using GC-MS confirmed two peaks were visible, one very small and one larger (Figure 212). Both peaks were confirmed as 5EAPB using the fragment ions, the molecular ion m/z=202 is shown in the mass spectrum in Figure 213. Fragment ions characteristic of 5EAPB are also shown; ’44, 72, 102 and 131’.

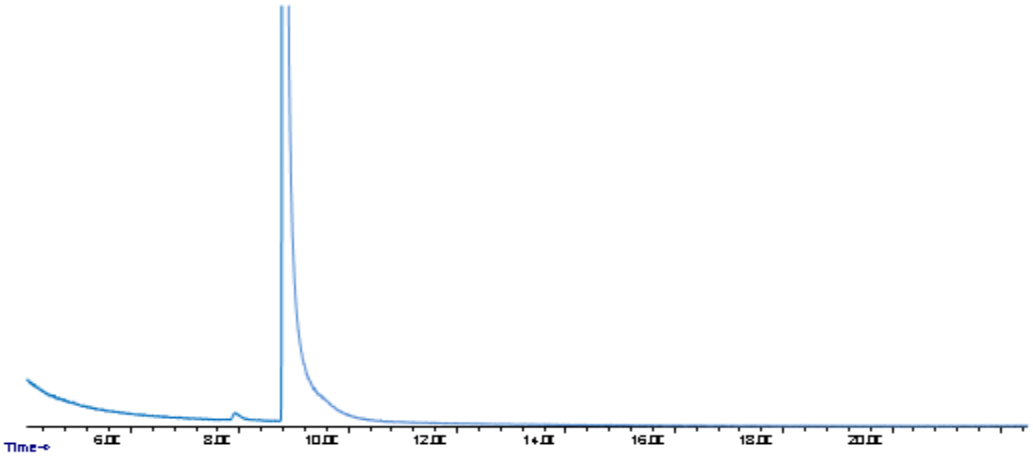


Figure 212: Chromatogram of 5EAPB.

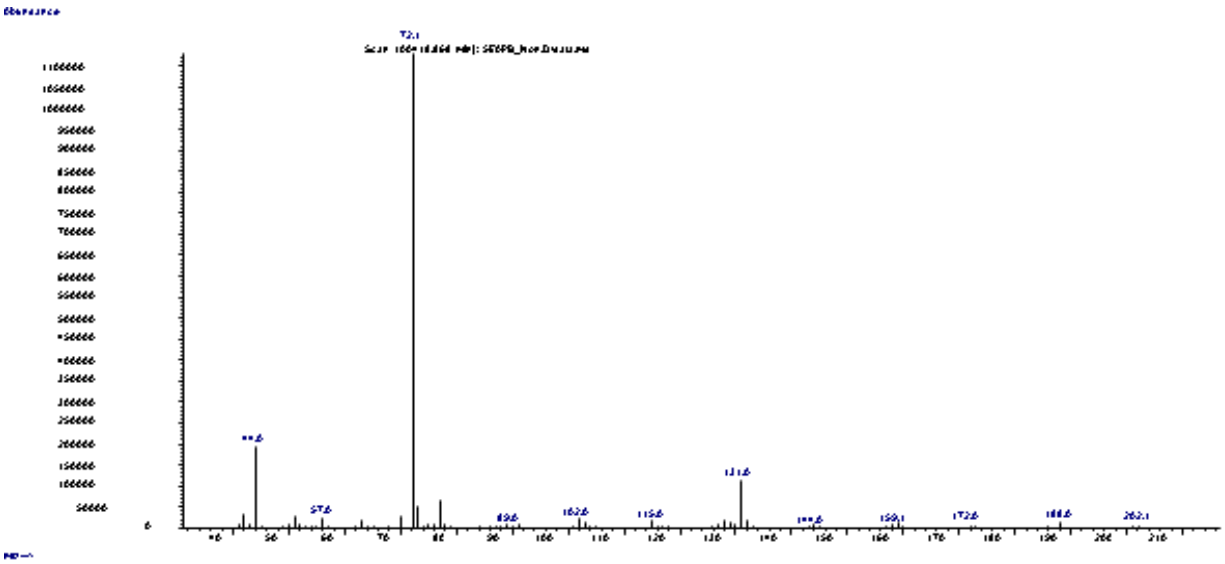


Figure 213: Mass spectrum of ion fragmentation shown by 5EAPB.

Conclusion

Substance confirmed as 5EAPB, explanation shown in section 7.3.1.10.

7.3.1.10 Explanation of fragment ions expected from APB compounds

The chemical structure of 6APB is shown in Figure 214. It consists of fused benzene and furan rings with an amine group. 5APB is a structural isomer of 6APB, in that the chemical formula is the same but the structure differs slightly. It is for this reason that differentiating between the two with GC-MS is not possible, as the same fragment ions would be detected.

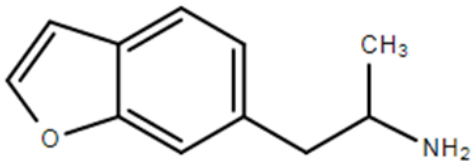


Figure 214: Chemical structure of 6APB.

During fragmentation the cleavage can occur resulting in the charge remaining on the amine group, resulting in an m/z= 44 as shown in Figure 215.

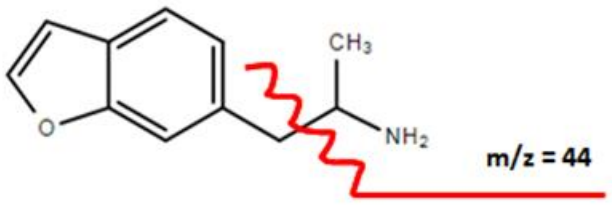


Figure 215: Chemical structure showing the position of breakage and resulting fragment m/z=44.

It the bond breaks and the charge remains on the benzene ring then the fragment ion m/z=131 will be detected (Figure 216).

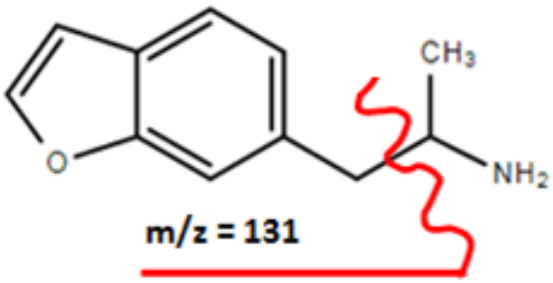


Figure 216: Chemical structure showing the position of breakage and resulting fragment m/z=131.

When the cleavage breaks in the position show in Figure 216, secondary cleavage can occur where a CHO group is cleaved resulting in m/z=102 fragment ion as shown in Figure 217.

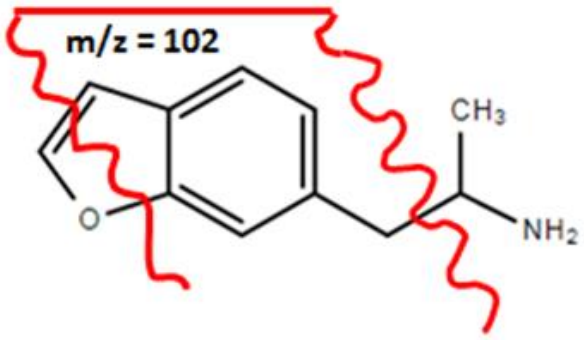


Figure 217: Chemical structure showing the position of secondary cleavage fragment m/z=102.

The substance purchased as '6APB' appears to show a similar mass spectrum to that observed previously during the analysis of Benzofury but not the same. The presence of 187 and 56 fragment ions indicates a structure with a mass difference of 12; this would suggest a carbon group is added. The suspected chemical structure of the received 6APB is show in Figure 218.

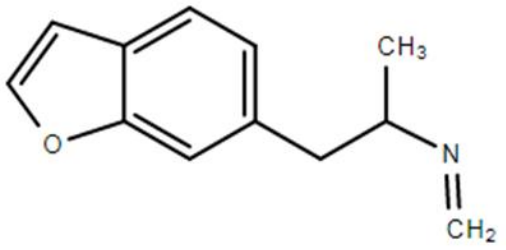


Figure 218: Suspected chemical structure of substance received as 6APB.

5EAPB is again chemically very similar to the previously discussed compounds (Figure 219); the primary amine is swapped with an ethyl group. Fragment ions m/z=44, 131, 102 are as shown for 6APB. The addition of fragment ion m/z=72 is observed and this is due to the addition of the ethyl group.

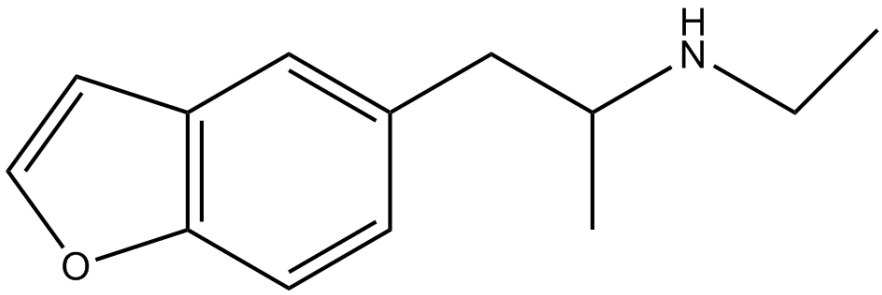


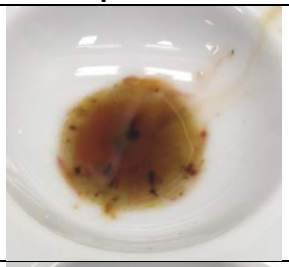
Figure 219: Chemical structure of 5EAPB.

7.3.1.11 AMT

Packaging can be seen in chapter 3. This drug was purchased from ViP-Legals.com and arrived in tablet form. One tablet was shown to be of violet colouring with a red tinge and the other was more red/pink in colour, these were uneven. The tablets themselves were well formed. Unfortunately, the pigment is shown to be very prominent during presumptive testing and colours are not as relatable.

Presumptive testing results are shown in Table 64. If the active compound of this substance is AMT then the reaction with Mandelin should show a deep greenish brown colour [327], this does appear as expected. Mecke should show a brown colouring which is again as expected. Marquis should exhibit a yellow/brown colour, this could be present with the pigment interfering, but this cannot be proved using this presumptive testing method. The three remaining reagents show very red colouring, it is suspected that this is due to the pigmentation of the tablet. Results for presumptive testing of AMT are inconclusive and require analysis using GC-MS.

Table 64: Presumptive test results for AMT.

Reagent	Colour produced	Notes
Mandelin reagent		Turns a dark/orange/brown colour.
Mecke reagent		Turned dark purple followed by a dark brown colouring.
Marquis reagent		A red/brown colour is observed with speckles.
Dille-Koppanyi reagent		Red/ pink colouring.
Robodope reagent		Very red instant colouring.
Ferric chloride reagent		Turned red instantly.

Analysis with GC-MS shows a number of peaks; this compound is not pure (Figure 220). The two peaks in highest abundance have been identified using the NIST library and the characteristic fragmentation ions observed within the mass spectrum.

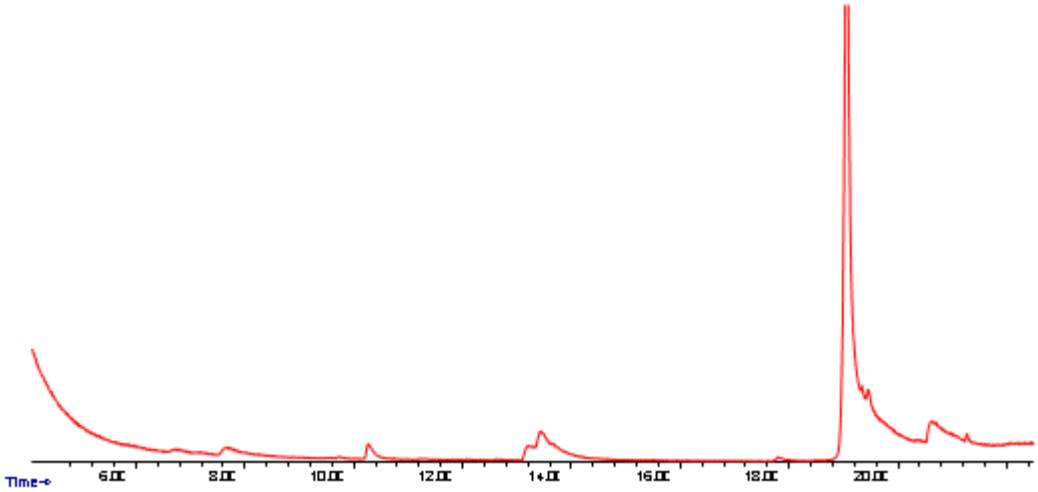


Figure 220: Chromatogram of AMT.

Fragmentation ions observed at 13.5 minutes are characteristic of 6IT. 6IT is a positional isomer of AMT and is a class B drug in the UK. It can be diagnosed with its molecular ion m/z=175 and distinguishing fragmentation ions ’44, 77,103, 130’.

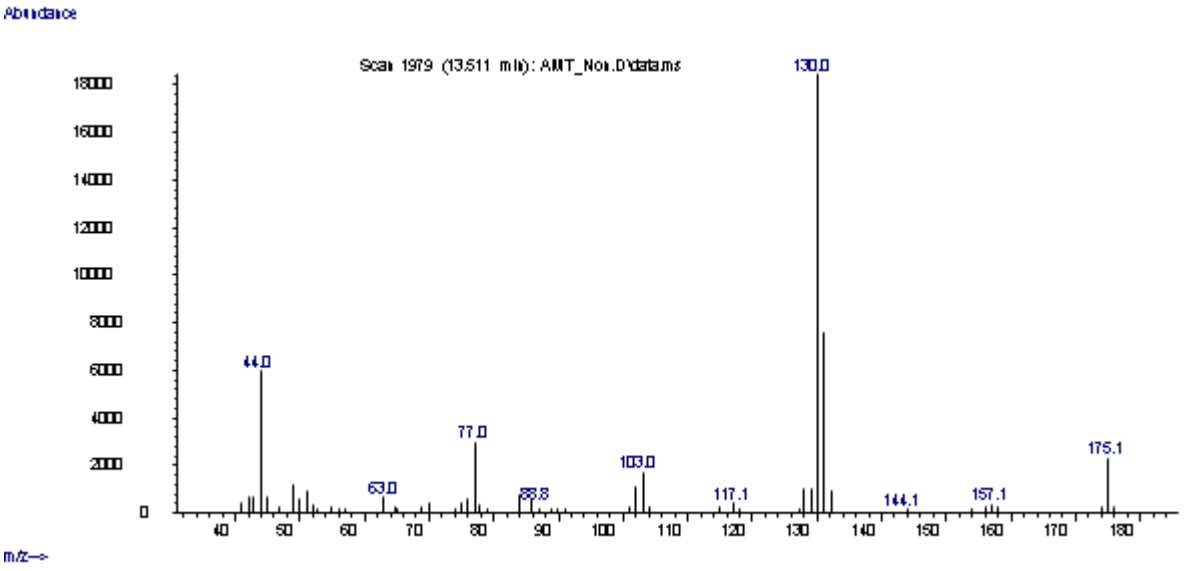


Figure 221: Mass spectrum of ion fragmentation shown by AMT at 13.5 minutes.

Fragmentation ions observed at 19.1 have been identified using the NIST library with an 83% match to 3-Methyl-1,2,3,4-tetrahydro-.gamma.-carboline which is a piperidine derivative with a molecular weight of '186'.

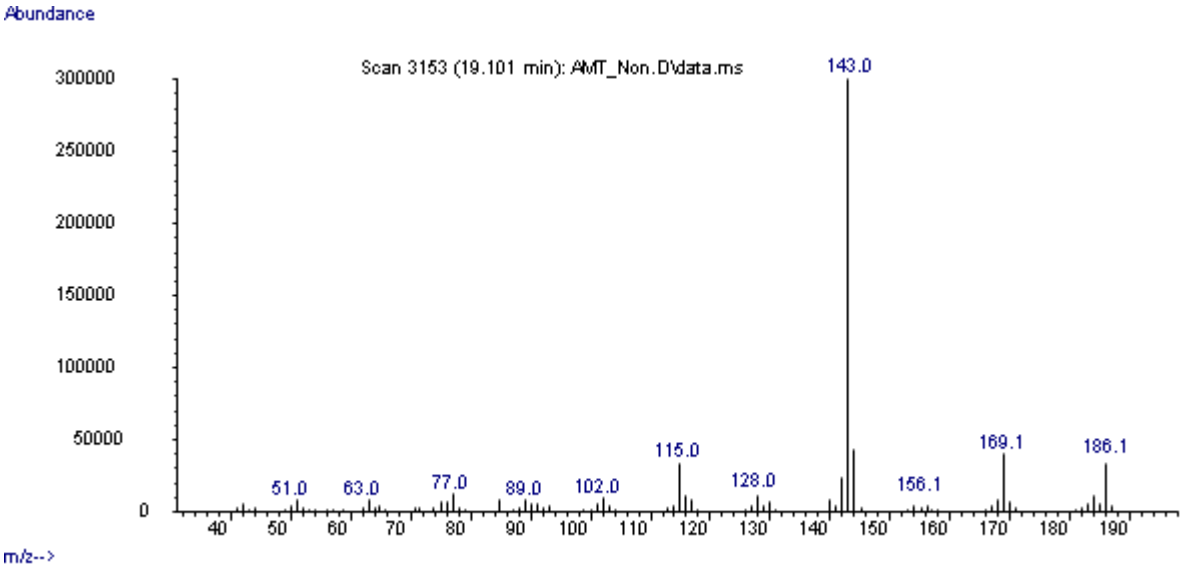


Figure 222: Mass spectrum of ion fragmentation shown by AMT at 19.10 minutes.

Identity of other peaks is unknown, a comparison was carried out with Caffeine, Benzocaine and Paracetamol as they were known adulterants of NPS, and these were all not found within AMT. A comparison chromatogram is shown in Figure 223.

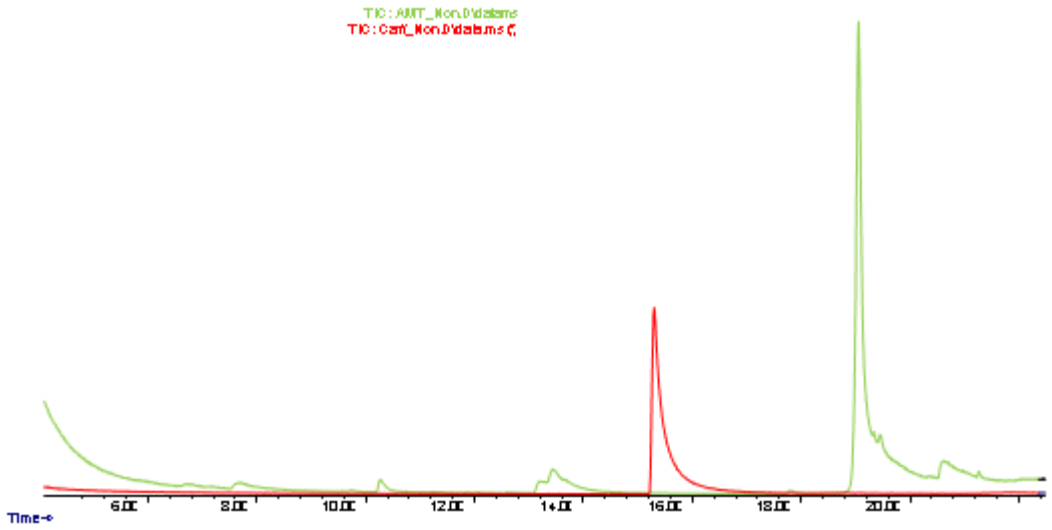


Figure 223: Comparison of AMT chromatogram with Caffeine.

Conclusion

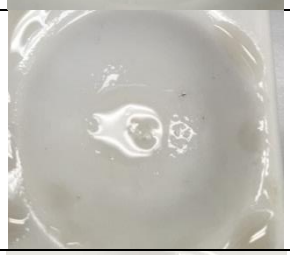
The compound sold as AMT was shown to contain a number of chemicals but not AMT, this is shown by comparing to reference spectra, the reader is directed to the appendix. 6IT a positional isomer of AMT was identified as well as a piperidine derivative, other peaks were not identified. The compound was impure.

7.3.1.12 Synthacaine

Synthacaine packaging is shown in chapter 3. This compound was purchased from legalhighsstore.co.uk; it arrived in powder form and was beige/off white in colour.

Presumptive test results are shown in Table 65. The drug did not appear to have much of a reaction with any of the reagents. Robodope showed a brown colouring, a positive reaction with Robodope reagent would usually be purple; this observed colouring could be due to the pigmentation of the powder. Presumptive tests are inconclusive and GC-MS analysis is required.

Table 65: Presumptive test results for Synthacaine.

Reagent	Colour produced	Notes
Mandelin reagent		Turns a yellow/light brown colour.
Mecke reagent		Fizzing, very slight yellow colouring observed.
Marquis reagent		No reaction observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		A golden yellow/ brown colour observed, this could be due to pigmentation of the powder.
Ferric chloride reagent		No reaction observed.

GC-MS analysis showed six peaks, this was not a pure product (Figure 224). The mass spectrum of each peak was observed to determine if it could be characterised.

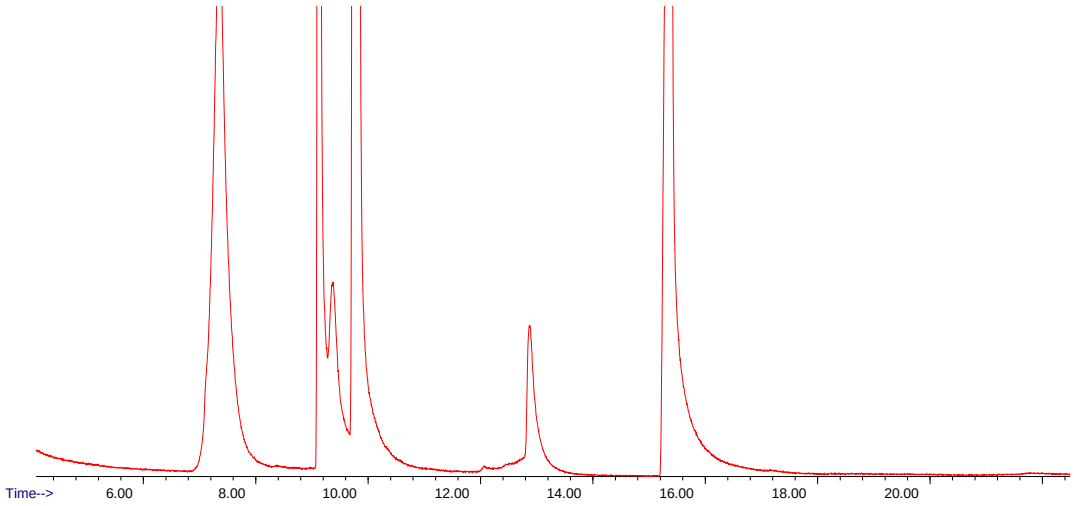


Figure 224: Chromatogram of Synthacaine.

Peak 1 did not show any match using the NIST library and fragmentation pattern was not recognizable (Figure 225).

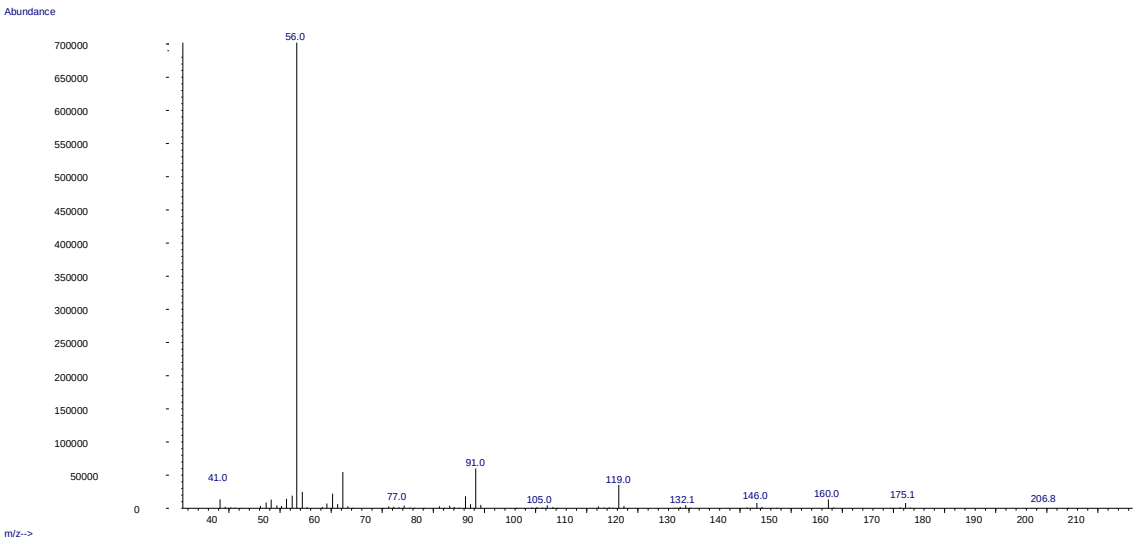


Figure 225: Mass spectrum of ion fragmentation shown by Synthacaine in peak 1.

Peak 2 did not show any match using the NIST library and fragmentation pattern was not recognizable (Figure 226), although recognition of the fragmentation ions could confirm Benzocaine presence.

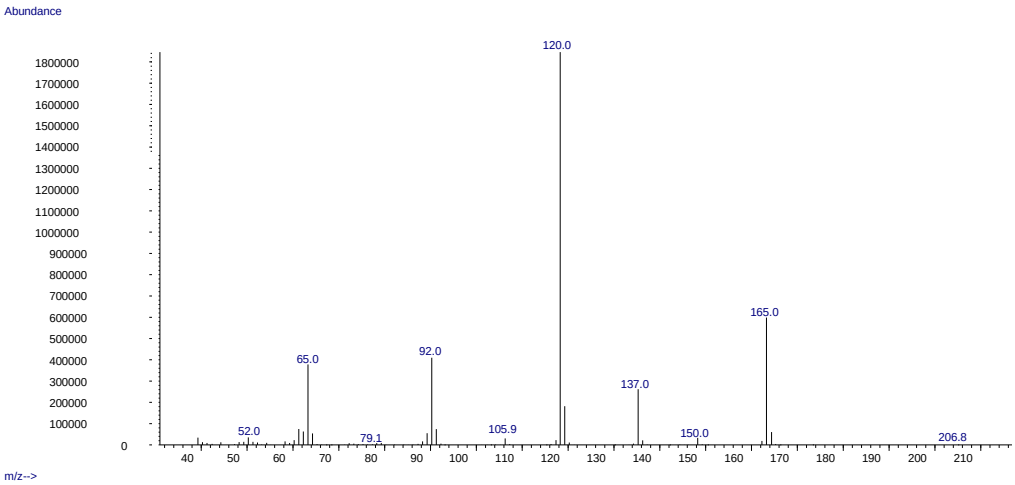


Figure 226 : Mass spectrum of ion fragmentation shown by Synthacaine in peak 2.

Peak 3 was identified using the NIST library and the fragmentation pattern of the peak as Benzocaine. The results observed during the presumptive testing are very similar to those shown for Benzocaine. The NIST library showed a 91% match and the molecular ion can be seen m/z=165 (Figure 227).

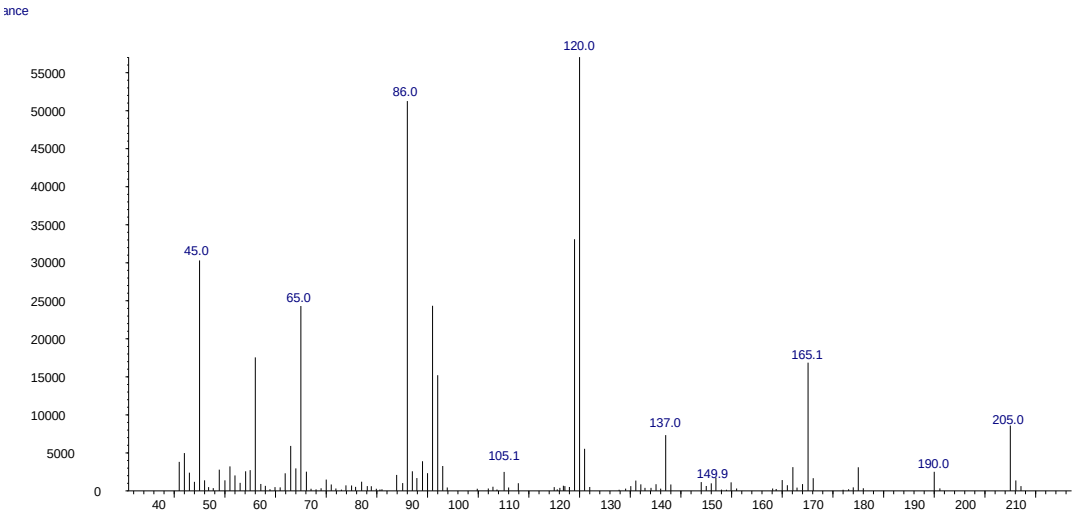


Figure 227 : Mass spectrum of ion fragmentation shown by Synthacaine in peak 3.

Peak 4 did not show any match using the NIST library and fragmentation pattern was not recognizable (Figure 228).

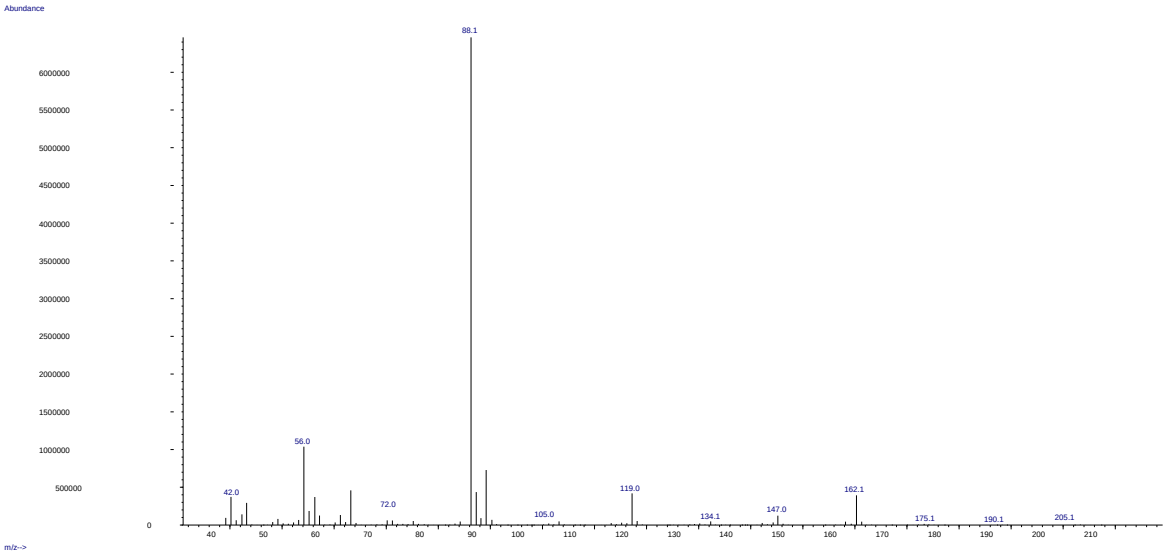


Figure 228 : Mass spectrum of ion fragmentation shown by Synthacaine in peak 4.

Peak 5 was identified using the NIST library as Toluic acid, which is a product used in the manufacturing process of polyethylene, it showed a match of 81% and the molecular ion m/z=205 is shown.

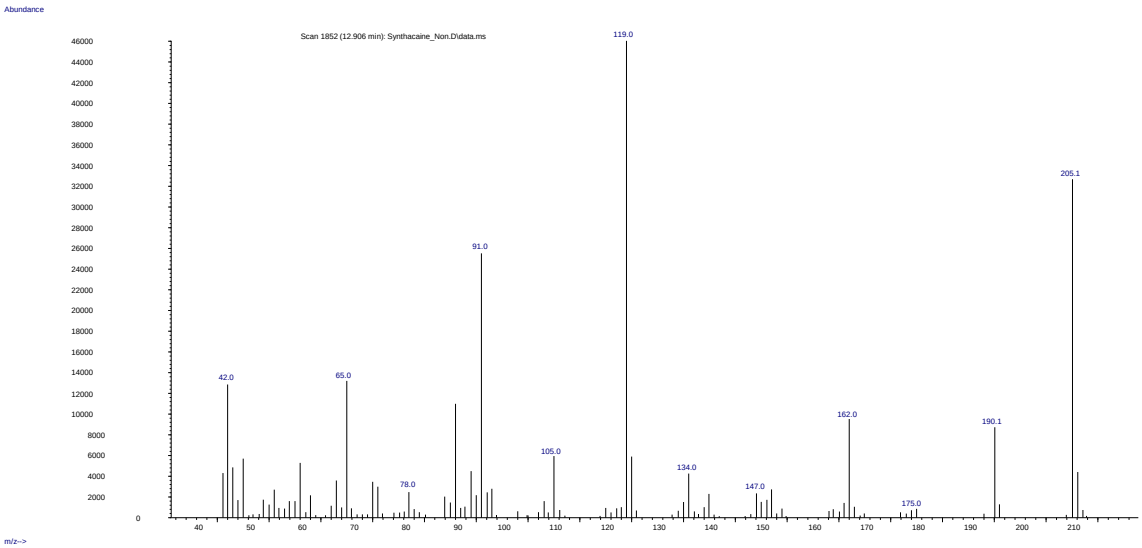


Figure 229 : Mass spectrum of ion fragmentation shown by Synthacaine in peak 5.

Peak 6 is identified using the NIST library and through the fragmentation pattern observed as Caffeine. The NIST library showed a 98% match and the molecular ion m/z=194 can be seen. Fragmentation ions often observed when Caffeine is present '165, 136, 109, 82, 67, 55 and 42' are all also observed within this mass spectrum.

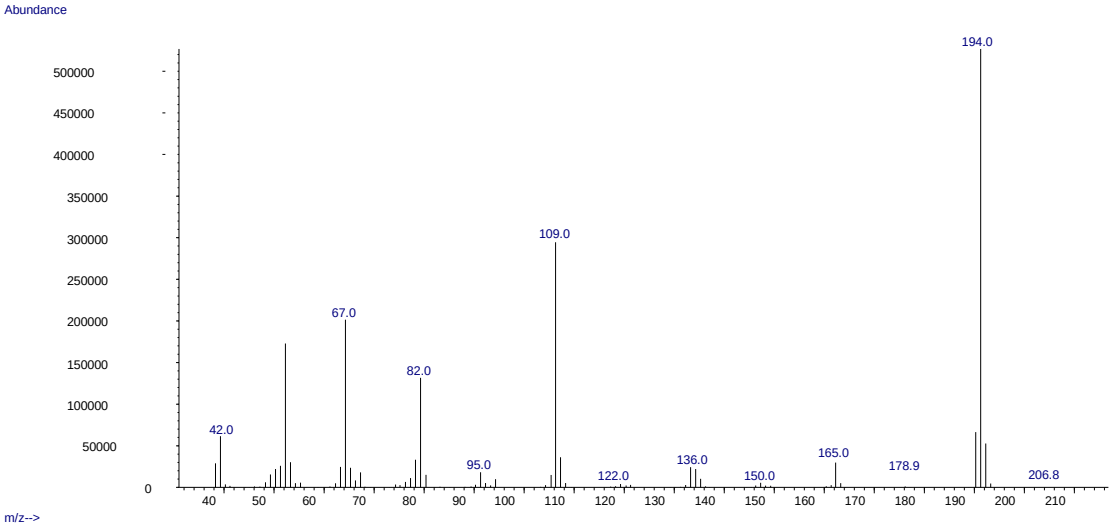


Figure 230: Mass spectrum of ion fragmentation shown by Synthacaine in peak 6.

Conclusion

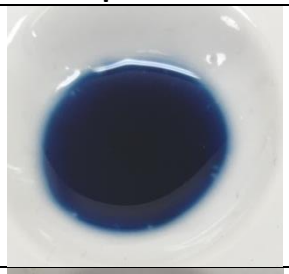
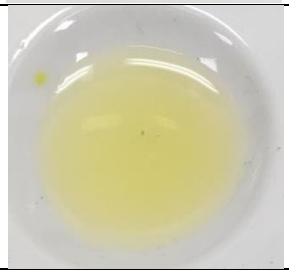
Contained within 'Synthacaine' are six components. 3 were not able to be identified. Benzocaine, Caffeine and Toluic acid were shown to be present.

7.3.1.13 Pink Panther

This substance was purchased from legalhighsstore.co.uk. The packaging for Pink Panthers is shown in chapter 3 and the pink translucent capsules found inside contained a white crystalline powder.

Presumptive testing results are shown in Table 66. Mandelin shows a blue colouring in reaction with the compound. If the drug present was MPA then this should turn a reddish-brown colour, 2AI however, would turn blue. The reaction of Mecke with 2AI is unknown; if the substance was MPA then the reaction colour would be black. Both MPA and 2AI turn dark brown with Marquis reagent, as observed in Table 66. A positive result can be seen using the Robodope reagent showing the presence of a primary amine such as MDA or amphetamine as opposed to a secondary amine. The presumptive testing shows the likelihood of 2AI presence within the NPS, this must be confirmed using GC-MS.

Table 66: Presumptive test results for Pink Panther.

Reagent	Colour produced	Notes
Mandelin reagent		Fizzing is noted, initially it turns green/yellow and then dark blue/green. After a further minute the colouring turns to blue.
Mecke reagent		A large amount of fizzing is noted, and a yellow colour is observed.
Marquis reagent		Fizzing noted. Colouring is a deep red/ orange/ brown.
Dille-Koppanyi reagent		No reaction observed, slightly pink on comparison.
Robodope reagent		Pink/purple colouring observed.
Ferric chloride reagent		No reaction observed.

GC-MS analysis of Pink Panther shows two visible peaks (Figure 231).

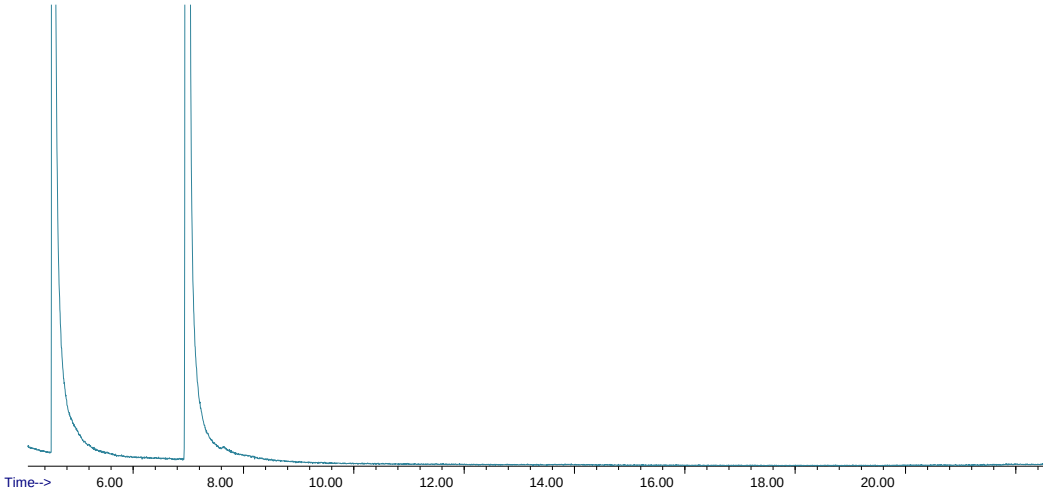


Figure 231: Chromatogram of Pink Panther.

Peak one (Figure 232) is identified using the presumptive testing results alongside the NIST library and characteristic fragmentation pattern observed as 2AI. A 98% match is shown with the NIST library; the presumptive tests had agreed with the initial identity, this is shown in the appendix. The molecular ion m/z=133 is observed as well as characteristic fragment peaks '116, 105, 91, 77, 63, 51 and 42'.

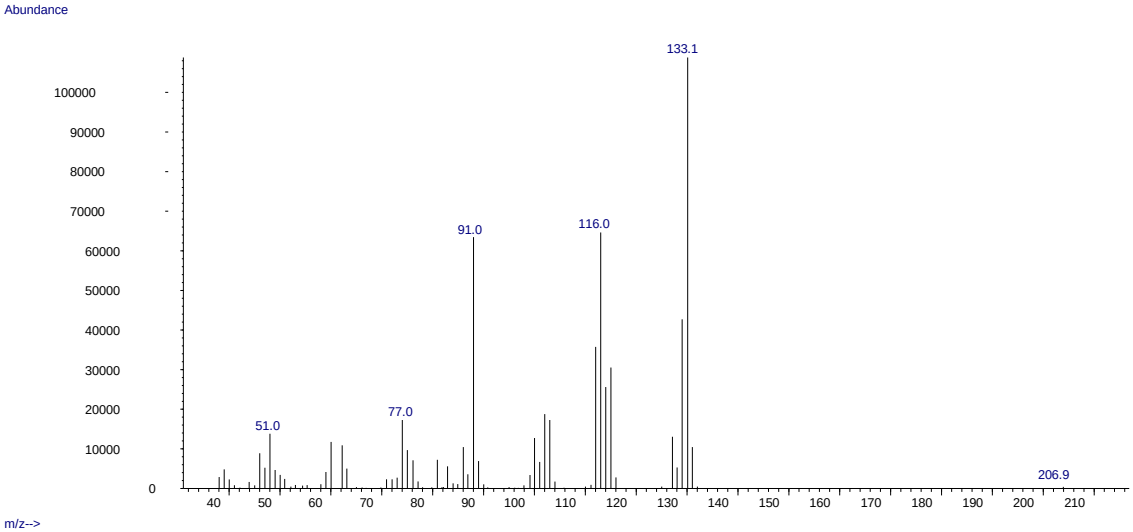


Figure 232: Mass spectrum of ion fragmentation shown by Pink Panther in peak 1.

Peak 2 did not show any match using the NIST library and fragmentation pattern was not recognizable (Figure 233).

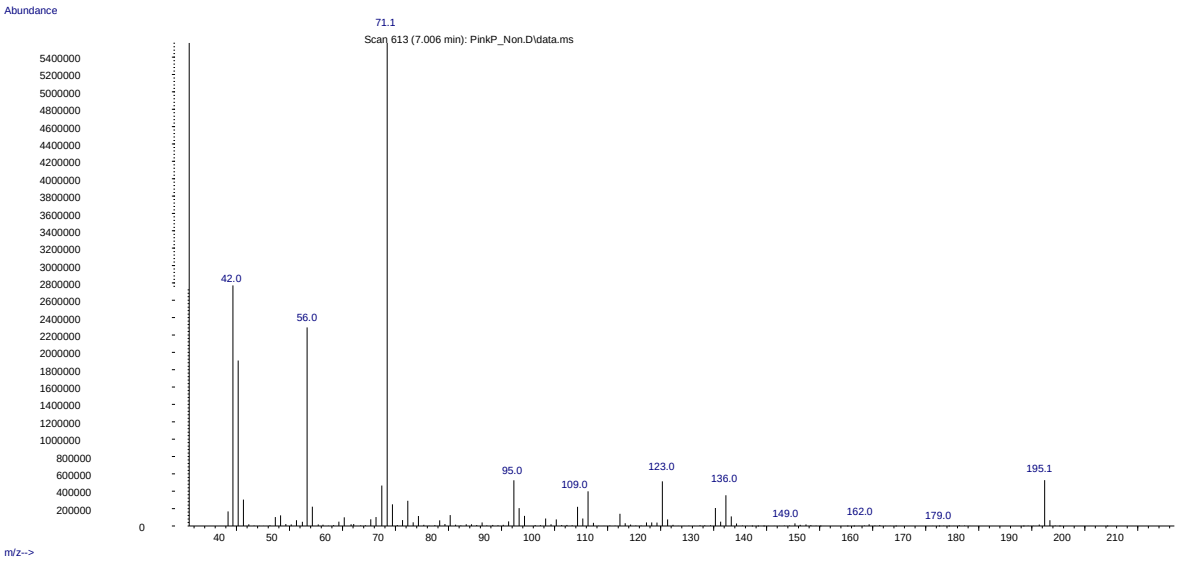


Figure 233: Mass spectrum of ion fragmentation shown by Pink Panther in peak 2.

Conclusion

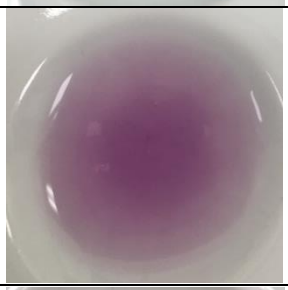
Pink Panther was suspected to contain 2AI; this was confirmed with presumptive testing followed by GC-MS analysis. One peak found during GC-MS analysis was not identified.

7.3.1.14 Ivory Wave

Ivory Wave was purchased from www.research-drugs.com. Packaging can be seen in chapter 3. Ivory Wave is a beige/off white powder.

Presumptive test results are shown in Table 67. A yellow colouring was observed in reaction to Mandelin reagent and also Ferric chloride reagent. The reasoning behind this is unknown. Robodope reagent has turned a pale purple perhaps signalling a primary amine is present although with negative results shown in Mecke and Marquis, this is slightly confusing. Presumptive testing is inconclusive and requires analysis using GC-MS.

Table 67: Presumptive test results for Ivory Wave.

Reagent	Colour produced	Notes
Mandelin reagent		Yellow colouring observed.
Mecke reagent		No reaction observed.
Marquis reagent		No reaction observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		Turned pale purple in colour.
Ferric chloride reagent		Slight yellow colouring observed.

GC-MS analysis of Ivory Wave shows only one peak (Figure 234), only one compound is present.

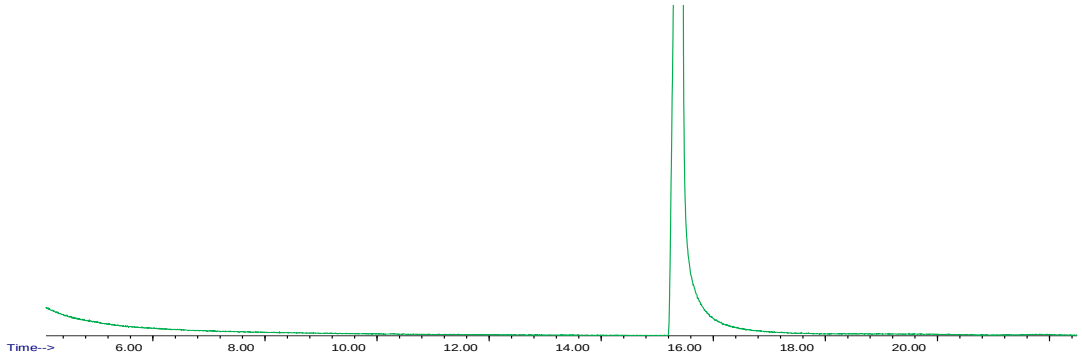


Figure 234: Chromatogram of Ivory Wave.

The identity of this peak was determined using a combination of the NIST library and fragmentation pattern observed. Ivory wave was shown to only contain Caffeine. The molecular ion m/z=194 can be seen. Fragmentation ions often observed when caffeine is present '165, 136, 109, 82, 67, 55 and 42' are all also observed within this mass spectrum.

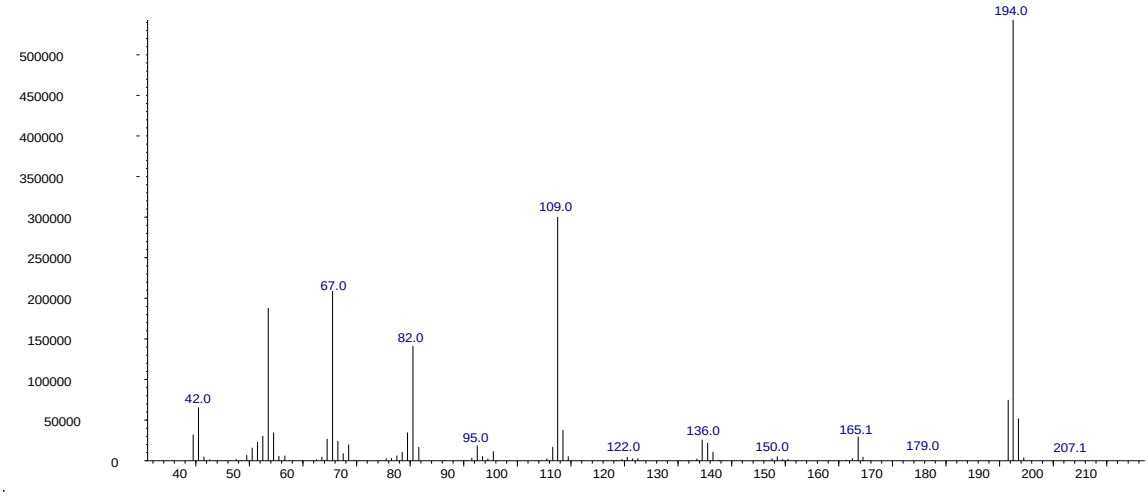


Figure 235: Mass spectrum of ion fragmentation shown by Ivory Wave peak.

Conclusion

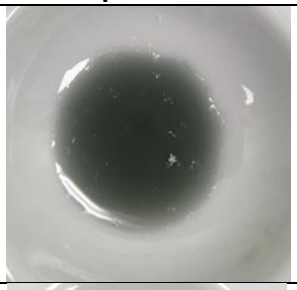
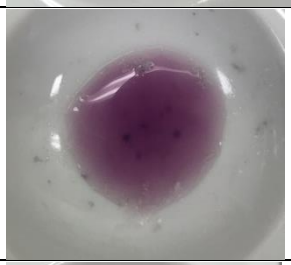
Ivory Wave is shown to only contain caffeine, though it is possible that other substances such as cellulose are present.

7.3.1.15 Blow

Blow was purchased from OfficialBenzofury.com and packaging can be seen in chapter 3. Blow appears to be a white crystalline powder.

Presumptive test results are shown in Table 68. If Blow contains MPA then mixing it with the Mandelin reagent would create a reddish-brown colour, instead a blue/green colour is observed. Mecke would show a black colour, instead a pale violet colour is observed. Marquis would show a dark brown colour and this is seen, however it is not indicative of MPA presence with the reactions shown with Mandelin and Mecke. Robodope shows a positive reaction with Blow indicating the presence of a primary amine. The presumptive tests are inconclusive and require further analysis using a confirmatory method like GC-MS.

Table 68: Presumptive test results for Blow.

Reagent	Colour produced	Notes
Mandelin reagent		Turned a blue/green colour.
Mecke reagent		Fizzing was observed and a pale violet colour seen, turning pale pink with a yellow ring around the rim.
Marquis reagent		Dark red/ brown colour observed.
Dille-Koppanyi reagent		No reaction observed.
Robodope reagent		Purple colouring observed.
Ferric chloride reagent		Yellow/brown colouring observed.

GC-MS analysis of Blow shows the presence of four peaks, three large and one smaller peak (Figure 236).

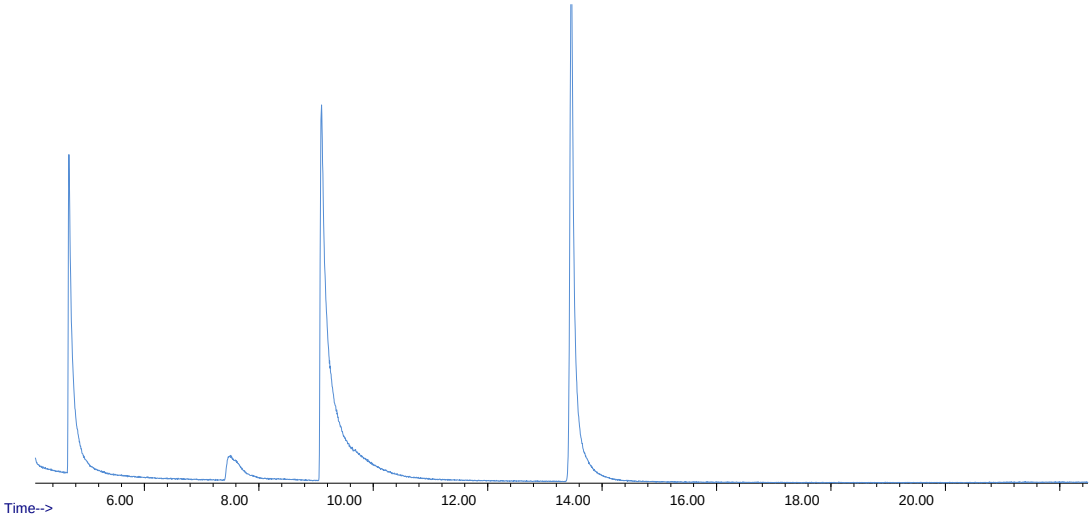


Figure 236: Chromatogram of Blow.

Peak one can be identified using the NIST library as Benzeneacetic acid with a 90% match. This is also known as Phenylacetic acid and is used in the manufacturing process of substituted amphetamines [326]. The molecular ion is visible m/z=164 (Figure 237).

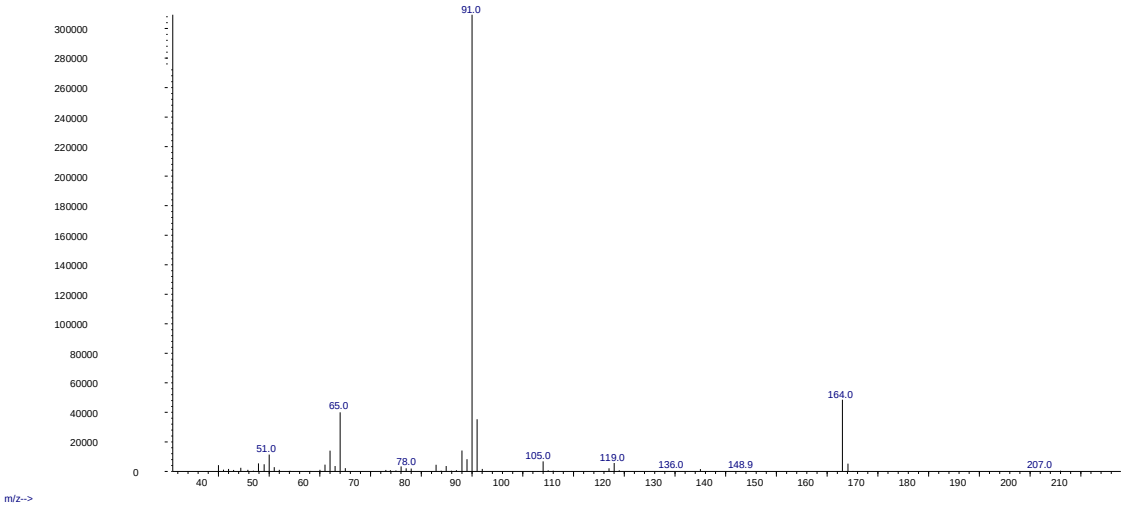


Figure 237 : Mass spectrum of ion fragmentation shown by Blow in peak 1.

Peak two can be identified using the NIST library as 4-[3-(Dimethylamino)propoxy]benzaldehyde with a 72% match. This compound is used in chemical synthesis [326]. Molecular ion m/z=207 can be observed. This is low library percentage match, so identity is tentative.

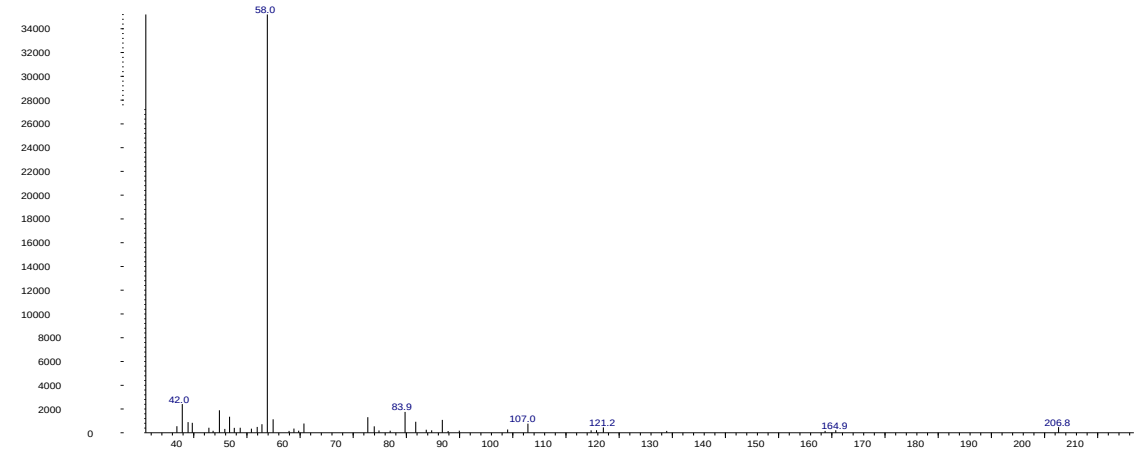


Figure 238; Mass spectrum of ion fragmentation shown by Blow in peak 2.

Peak 3 was identified using the NIST library and the fragmentation pattern of the peak as Benzocaine. The results observed during the presumptive testing are very similar to those shown for Benzocaine. The NIST library showed a 91% match and the molecular ion can be seen m/z=165 (Figure 239).

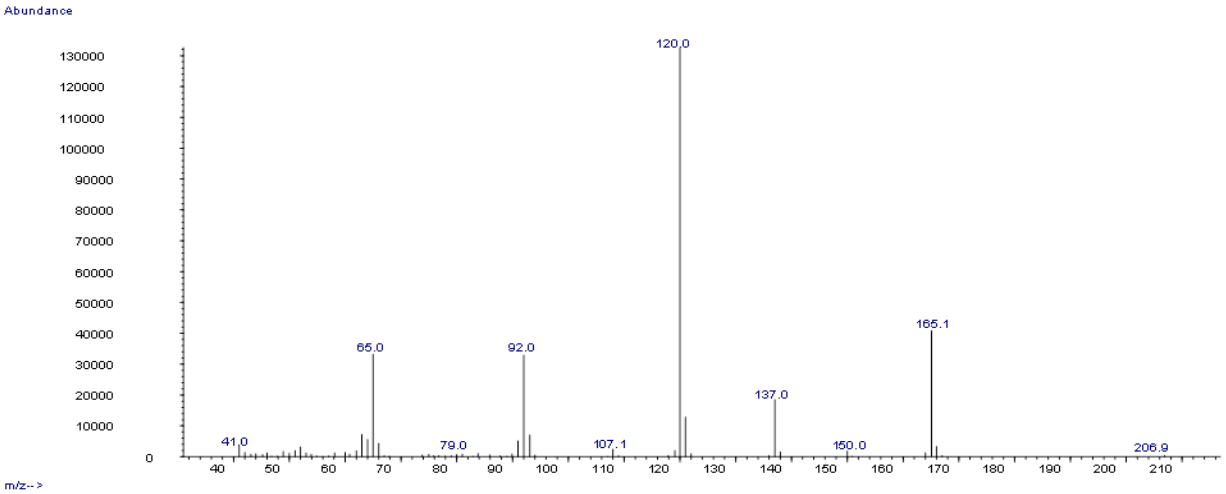


Figure 239: Mass spectrum of ion fragmentation shown by Blow in peak 3.

Peak 4 was identified again using the NIST library as Methylphenidate with an 86% match. This compound is often known by its trade name 'Ritalin' and is a class B stimulant in the UK (Figure 240).

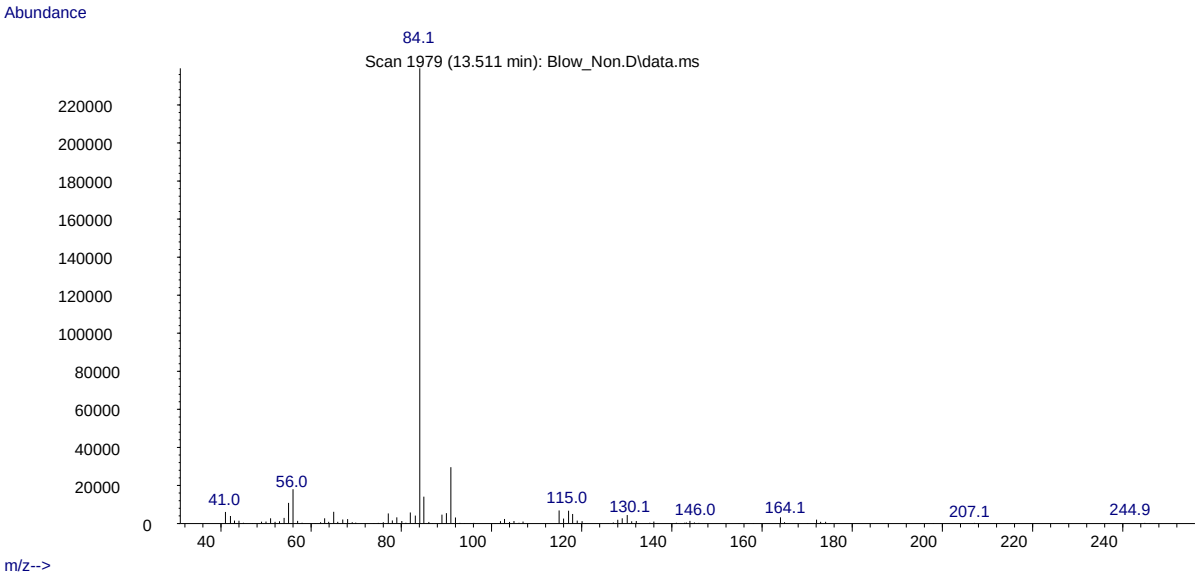


Figure 240 : Mass spectrum of ion fragmentation shown by Blow in peak 4.

Conclusion

The analysis of Blow determined that it contained four components, Phenyl acetic acid that is used in the manufacturing process of substituted amphetamines, a tentative identification of 4-[3-(Dimethylamino)propoxy]benzaldehyde, which is used in chemical synthesis, Benzocaine, a suspected common adulterant of NPS and Methylphenidate, a class B stimulant.

7.3.1.16 MDA

MDA was sourced from a reference collection from Kinesis.

The chromatogram from GC-MS analysis is shown in Figure 241. Only one peak is visible, it is a pure compound.

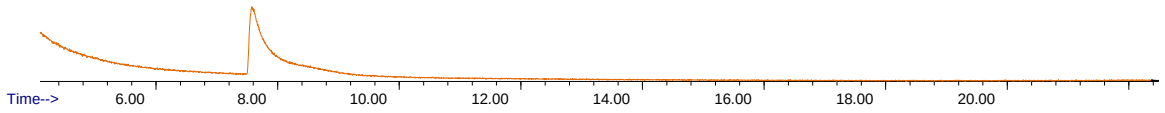


Figure 241: Chromatogram of MDA.

The mass spectrum of MDA is shown in Figure 242, molecular ion '179' is visible.

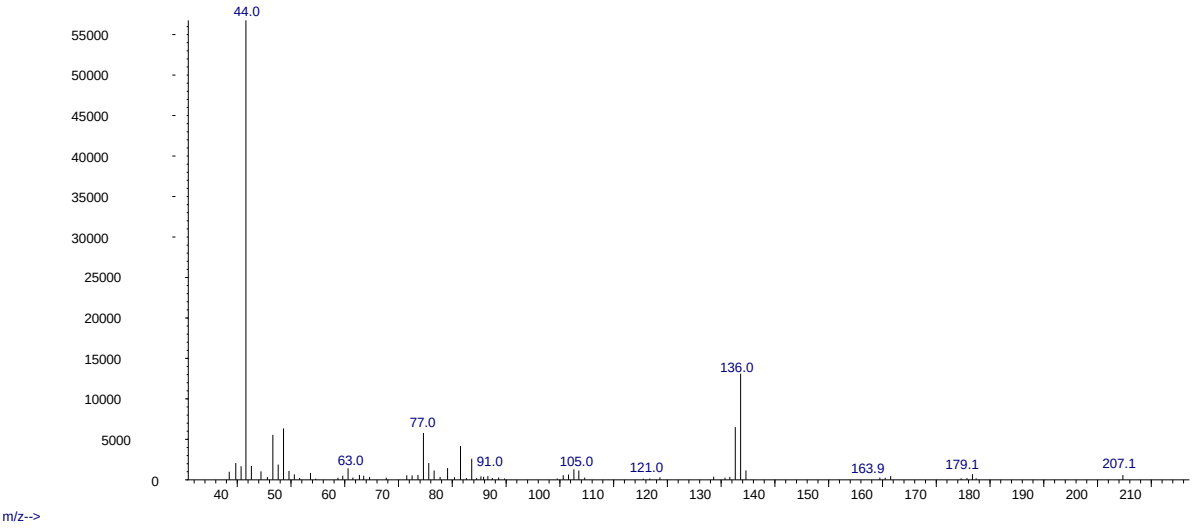


Figure 242: Mass spectrum of ion fragmentation shown by MDA.

Conclusion

The chemical composition of MDA can be confirmed.

7.3.2 Internal analysis Results

7.3.2.1 Testing

Results from the testing of NPS analysis from larval internals was shown to be successful. The retention time of each NPS is known from prior testing, this increases ease of analysis. Once a peak was detected in an area in accordance to the known retention time, the mass spectrum was examined for characteristic fragmentation ions. It must be noted that these are not the same ions as shown in NPS analysis as derivatisation has taken place. A larval sample with 'Ivory Wave' is shown in Figure 243. Analysis of 'Ivory Wave' earlier in this chapter, showed that this sample contained caffeine.

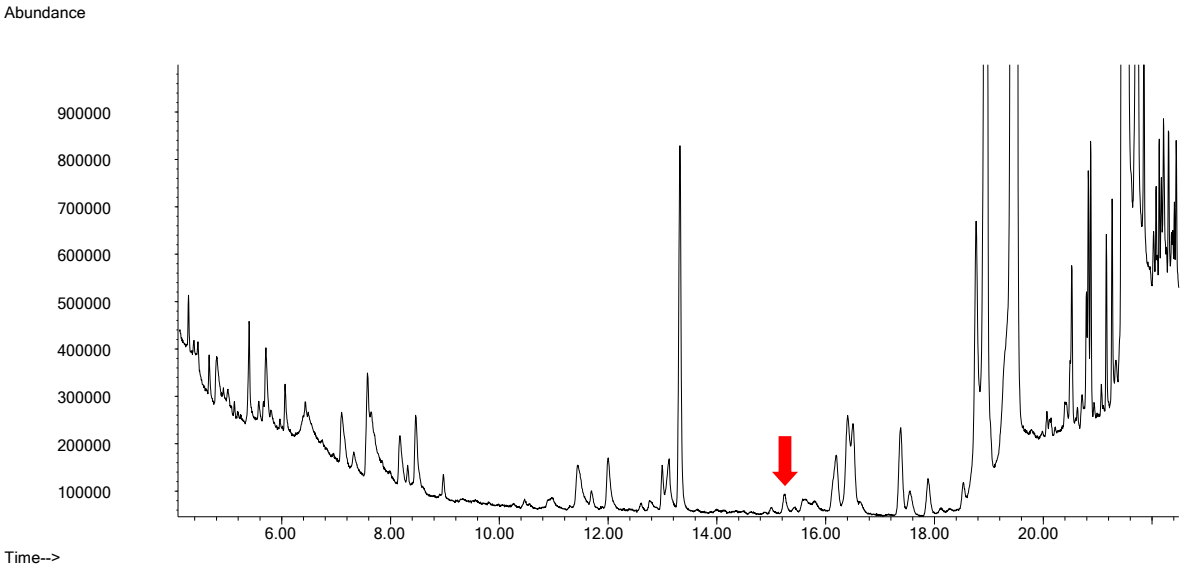


Figure 243: Chromatogram showing internal sample spiked with Ivory Wave prior to preparation.

The mass spectrum of the internal sample containing 'Ivory Wave' (thought to be just caffeine) was examined for characteristic peaks; these are shown in Figure 244.

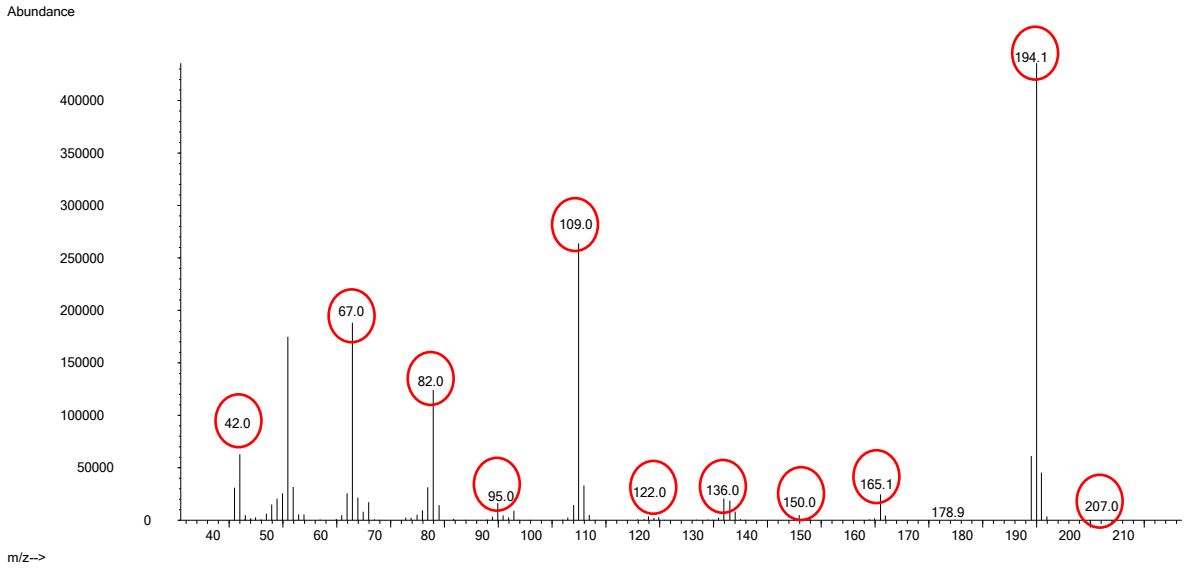


Figure 244: Further investigation showing mass spectrum of peak shown in Figure 243, confirming the presence of Ivory Wave.

An example is shown in Figure 245 where a larval sample was spiked with 5EAPB, retention time of derivatized 5EAPB is known from previous testing shown in this chapter.

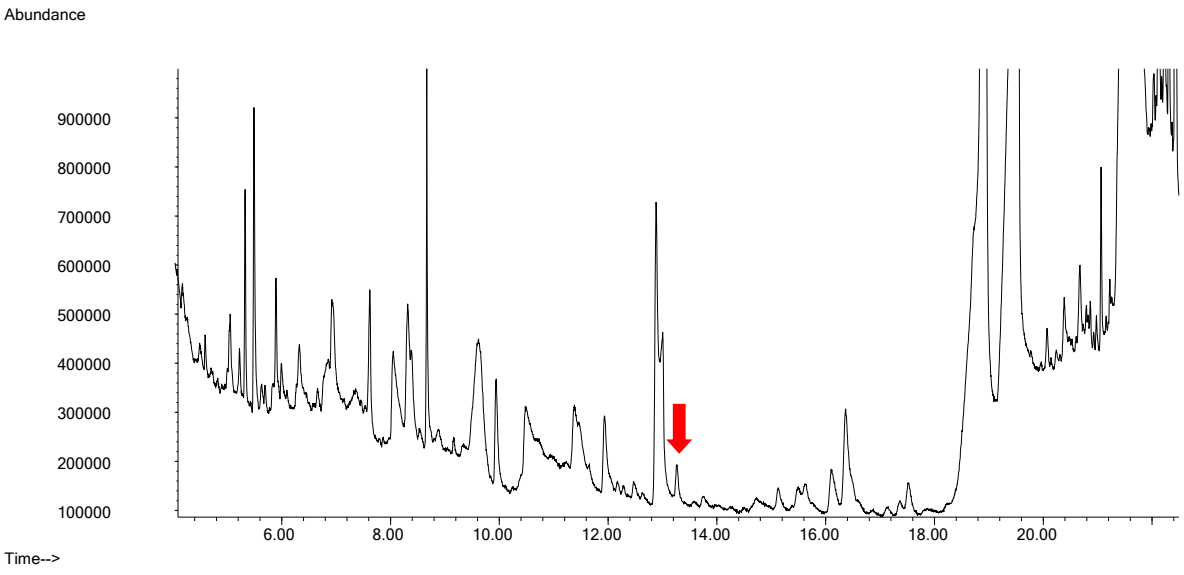


Figure 245: Chromatogram showing internal sample spiked with 5EAPB prior to preparation.

Once the peak was detected, the mass spectrum was once again analysed for characteristic fragmentation ions of derivatized 5EAPB. These are shown circled in Figure 246.

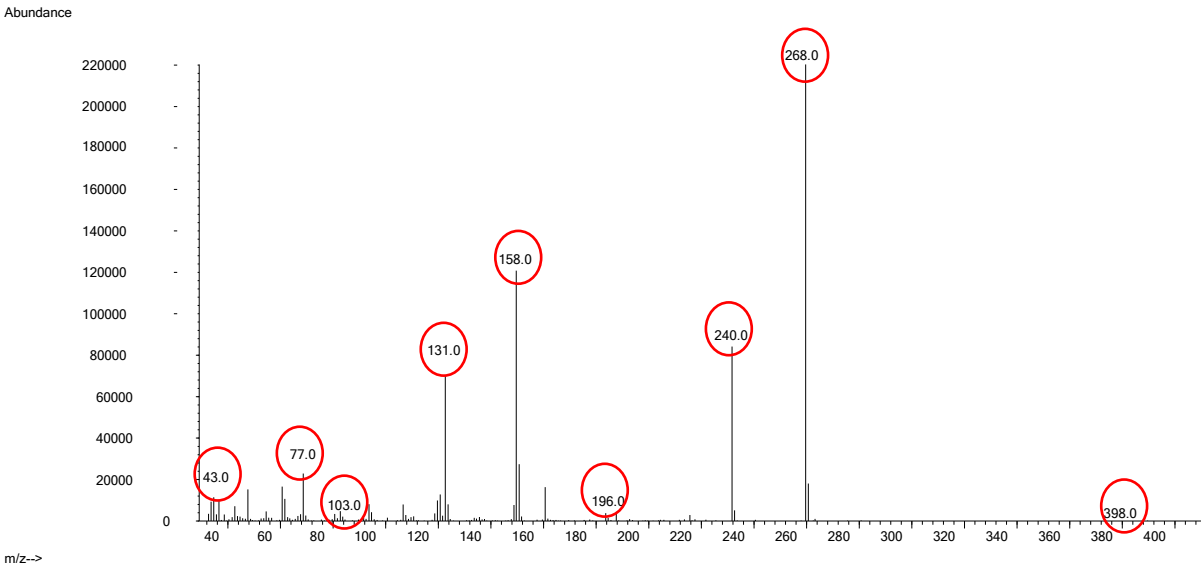


Figure 246: Further investigation showing mass spectrum of peak shown in Figure 243, confirming the presence of 5EAPB.

7.3.2.2 Internal Larval samples

Extracts were firstly analysed using the known retention times of the drugs shown within this study, once peaks were identified, the mass spectrum was analysed to determine if diagnostic ions were present.

An example of the internal chromatogram of a larval sample that has ingested 5EAPB is shown in Figure 247 compared with derivatized 5EAPB.

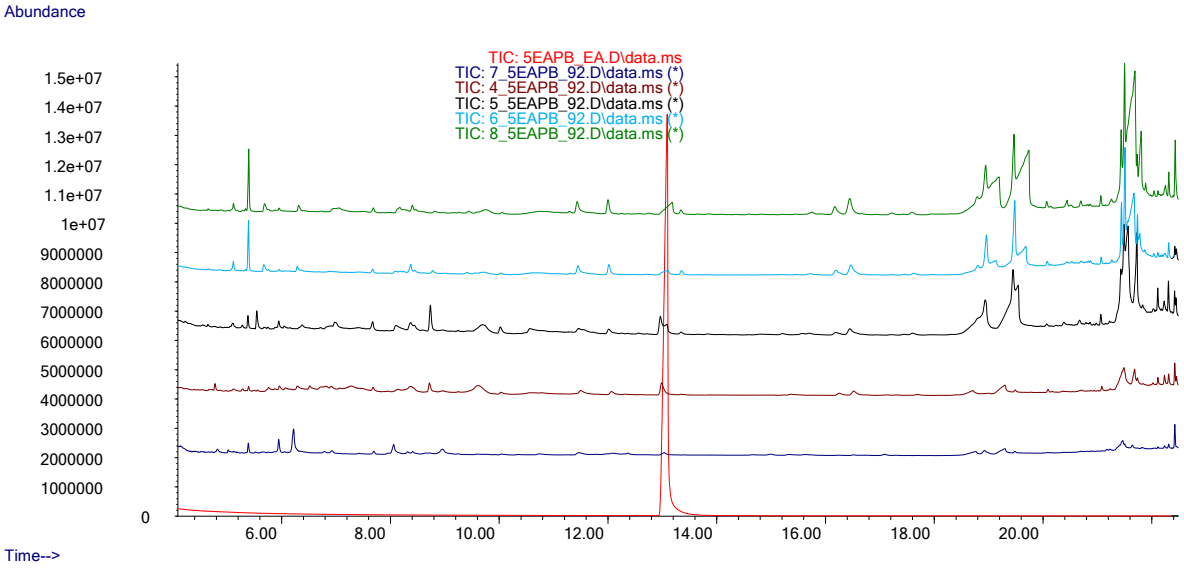


Figure 247: Chromatogram of internal larval extracts from '5EAPB' population.

The peak is analysed using the mass spectrum to determine diagnostic ion presence, the diagnostic ions used to identify the existence of 5EAPB are shown circled in Figure 248.

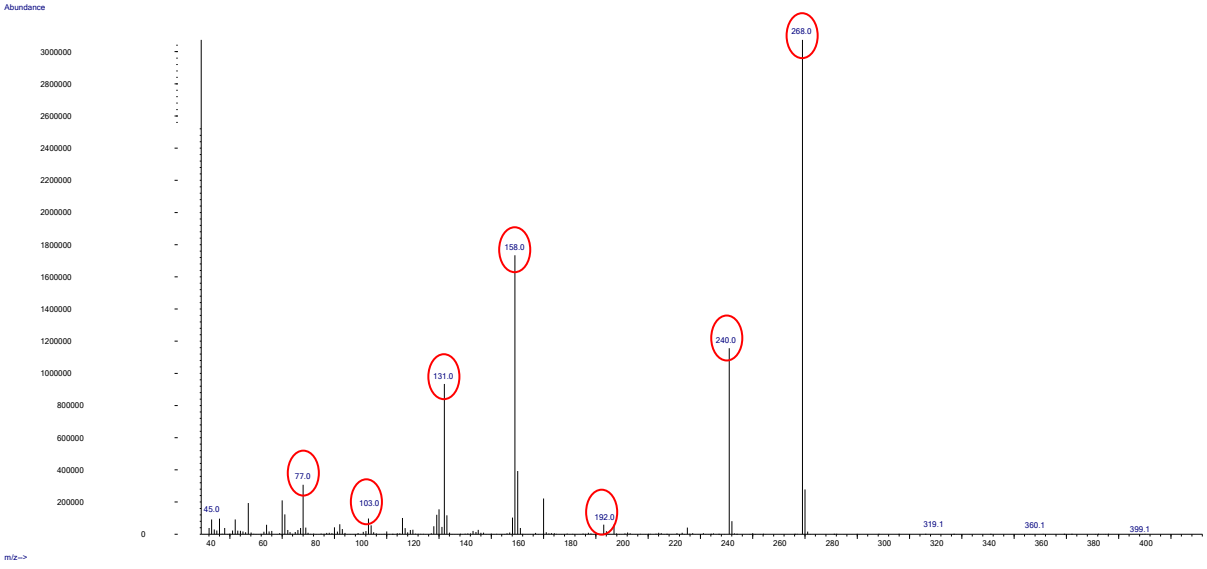


Figure 248: Mass spectrum of suspected '5EAPB' peak from larval extraction with diagnostic ions circled.

An example of the internal chromatogram of a larval sample that has ingested ‘Benzofury Beige’ (analysed as 6APB/5APB) is shown in Figure 249 with derivatized ‘Benzofury beige’ for comparison.

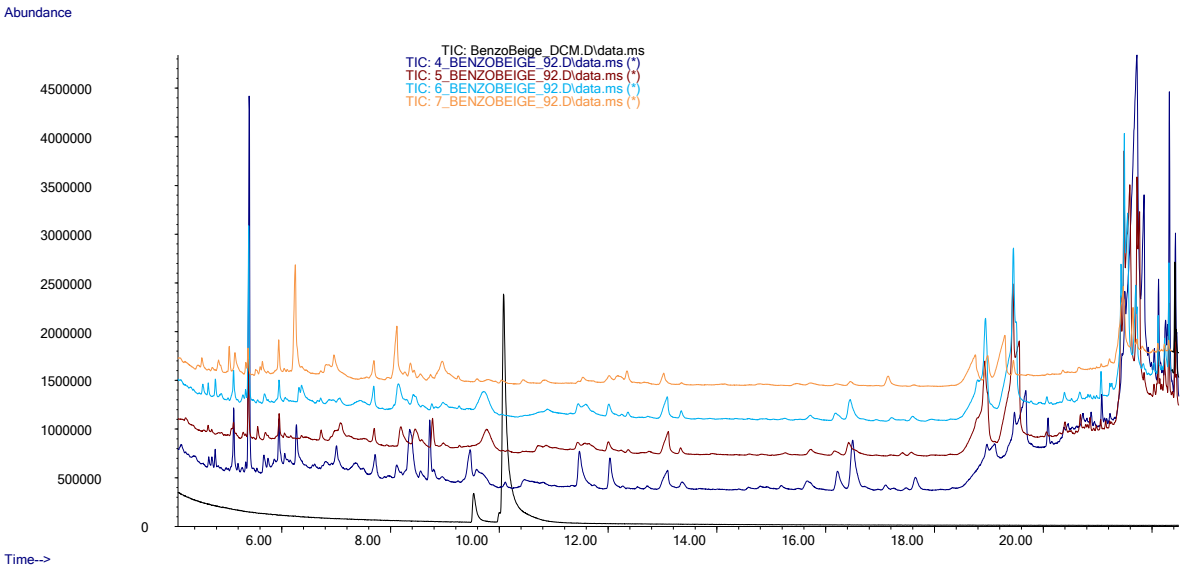


Figure 249 : Chromatogram of internal larval extract from ‘Benzofury Beige’ population.

The peak is analysed using the mass spectrum to determine diagnostic ion presence, the diagnostic ions used to identify the existence of ‘Synthacaine’ (analysed as a six component mixture) are shown circled in Figure 250.

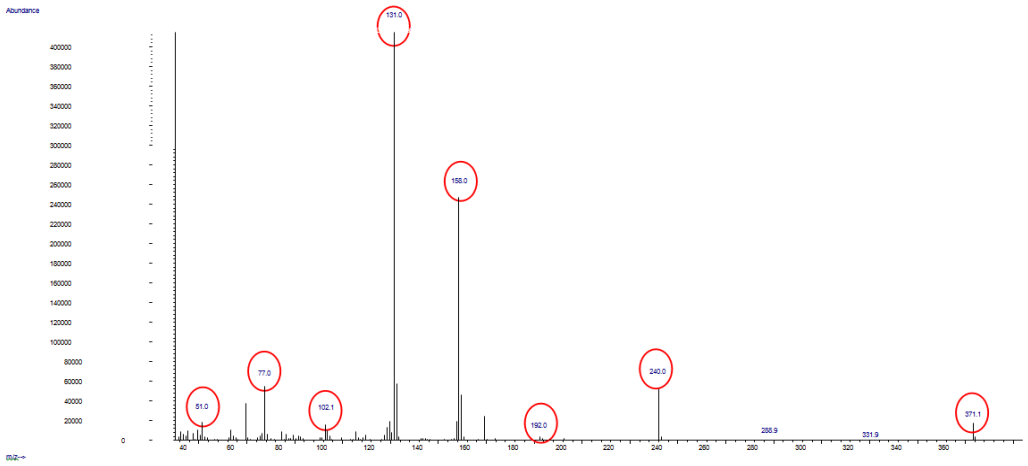


Figure 250 : Mass spectrum of ‘Benzofury Beige’ peak from larval extraction with diagnostic ions circled.

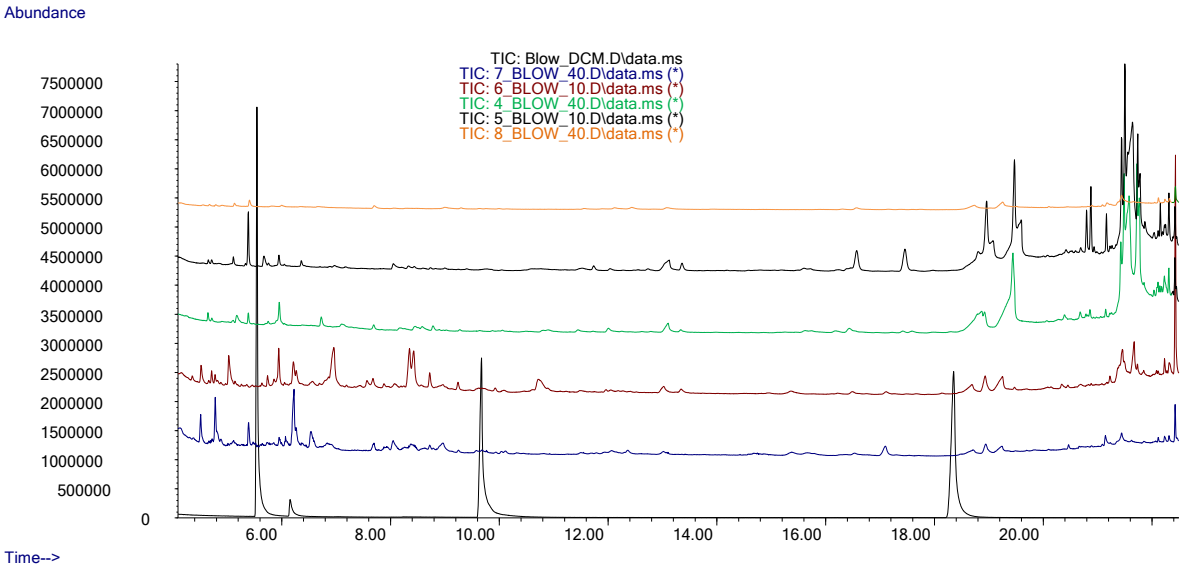


Figure 251: Chromatogram of internal larval extract from 'Blow' population.

Internal larval samples were analysed daily to determine if the drugs provided to the blowfly larvae were present in the larval extract on different sampling days. This would also allow confirmation that the effects observed on developmental rate was due to NPS ingestion. The samples were only analysed for the parent drug as the metabolic breakdown of these NPS is unknown and beyond the scope of this research. Results are tabulated below in Figure 252, red shows that the drug was not detected in any of the larval samples from that sampling day, black shows that no samples were available due to pupariation of the population. Samples were only analysed in their larval form not pupae or adult blowfly. It is suggested that drugs are most likely to be present in larval samples actively feeding [146] on the diet, as explained previously in this chapter. This was the focus of this section of the project. Green shows where the drug has been identified in at least one of the larval samples from that sampling day.

No drugs are detected in Days 1-3 of sampling, it can be suggested that the amount of drug ingested by the larvae has not been allowed sufficient time to build up above the detection limit of the GC-MS. Previous data within this thesis has also suggested that palatability of the diets has been affected by some drug concentrations; it is possible that the larvae have not ingested large amounts of the drug containing diet by these developmental days. Drugs initially appear at day 4 in one sample, the amount of food in the crop of the larvae at this stage will be increased. It can be suggested from the data shown within this thesis that third instar larvae are most likely to show drug presence. The results vary dramatically, and it is likely that this is due to differences in drug composition, elimination and metabolism rates. At present this information is unknown. It was not possible to determine all the drugs contained in this research at larval stage, this could be because the drug is not present and has instead been eliminated by the larvae or perhaps the drug has been metabolised by the larvae and the compound now present is unknown. There is also potential that the drug is in concentrations too small to be detected. Caffeine, 'Blow', '6APB', 'Benzofury' Blue, 'Benzofury' Green, 'Synthacaine', 'Pink Panther', 'Ivory Wave', MDA and 'AMT' are not identified in any sampling day (see previous analysis for contents of these compounds). '5EAPB' is detected for the largest number of sampling days, from day 5 to day 7, pupariation then occurs after day 8. 'Benzofury' Blue, Green and Beige along with '6+5APB' have been shown to be similar in composition which would suggest a similar pattern within the larval internal samples, this is not seen, 'Benzofury' Beige is the only one of these to be present, and this is likely due to concentration of the active compounds or detection limit capability of the GC-MS. The sampling day just prior to pupariation are likely to show the drug in the highest concentration, this is because larvae which are actively feeding store ingested food in

their crop; this expands during periods of ingestion. At this point the drug would be concentrated within the crop and it could be perceived that a larger concentration is present. The crop is also known to empty very quickly upon conclusion of feeding [39], this would potentially result in no drug detected within the insect sample. Absence of chemicals from the larvae does not definitively indicate absence from the food source [118] as shown here. All larvae were washed prior to collection as specimens; this was to ensure realistic drug concentrations were detected. One study found that samples which were not washed before analysis showed significantly higher concentrations compared with those samples which were washed correctly [119], this shows that for successful quantitative analysis, at any level, a washing protocol should be followed [130].

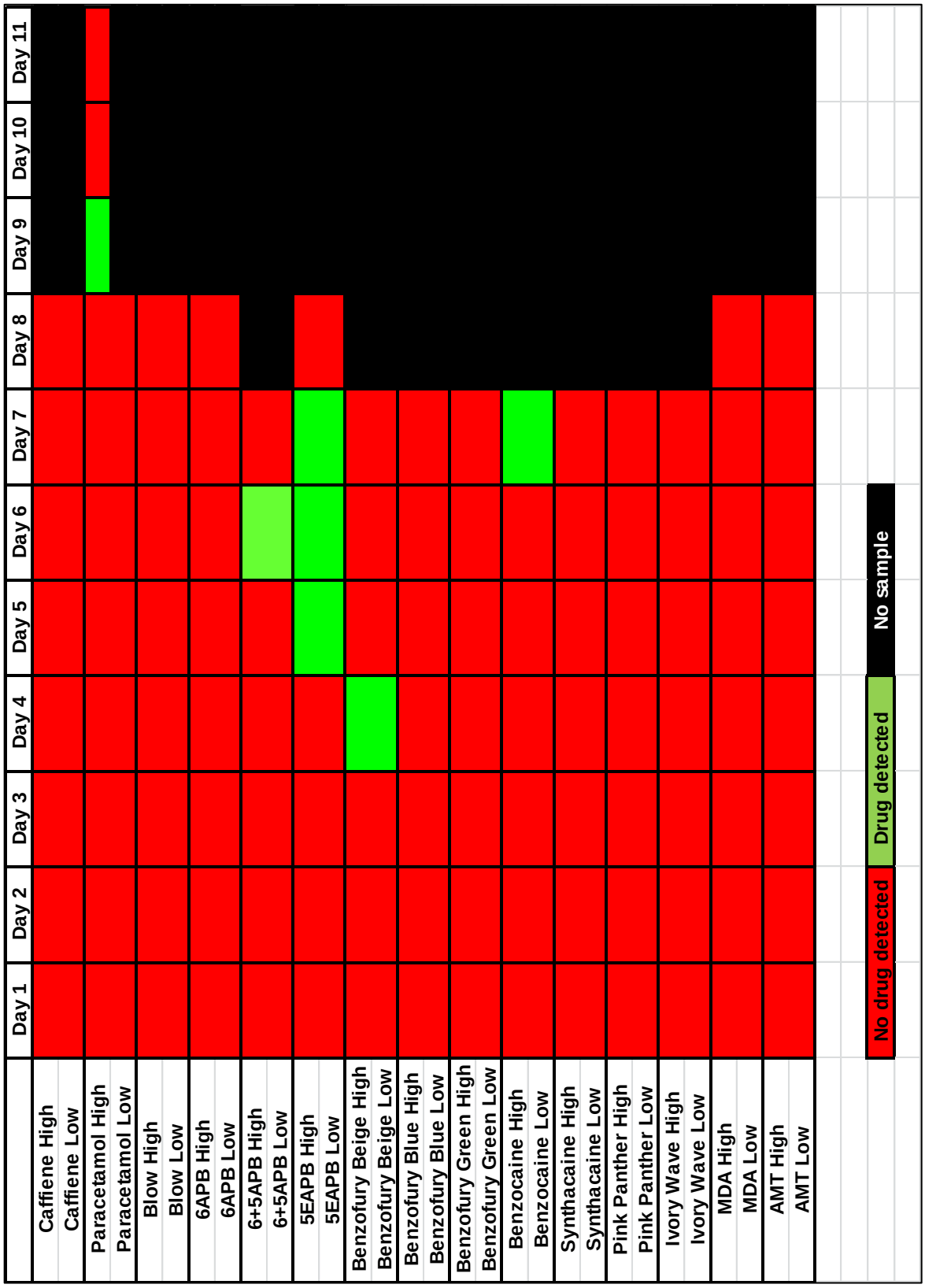


Figure 252 : Tabulated results showing identification of drugs within larval samples at different sampling days. Sample names are shown – please note analysis of these drugs is shown earlier in this chapter.

Conclusion

Studies have shown varying levels of usefulness in quantitative analysis of drugs in insect specimens. The metabolic pathways, elimination rate and initial concentration alongside species will all vary the presence of these drugs. Only five of the drugs analysed within this study were detected within the internal larval samples in at least one sampling day. Reasoning behind these differences has been deliberated. Further research into the breakdown of these chemicals is necessary in order to more accurately determine the limiting factors when analysing internal samples.

7.4 Overall Conclusion

Firstly, all drugs of interest to this study were analysed to determine their composition, this was carried out by initially using presumptive testing and then confirmatory analysis (GC-MS). Caffeine, Paracetamol, Benzocaine and MDA were all confirmed as pure compounds and diagnostic fragmentation ions discovered. All three batches of 'Benzofury', (Beige, Blue and Green) were confirmed as pure 6APB and diagnostic fragmentation ions shown. '6APB' could not be confirmed as 6APB, it is apparent that a substance very similar in composition is present, the potential structure has been shown. '6 and 5APB' was shown to be either 6APB or 5APB or a mixture of both, determination is not possible, the ions to identify this compound were identified. '5EAPB' was identified and showed to be as expected; this can now be identified using characteristic ions. The compound sold as 'AMT' was shown to contain a number of chemicals but not 'AMT'; this

was confirmed with reference material shown in the appendix. 6IT a positional isomer of AMT was identified as well as a piperidine derivative, other peaks were not identified. The compound was impure. Contained within 'Synthacaine' are six components. 3 were not able to be identified. Benzocaine, Caffeine and Toluic acid were all shown to be present. 2AI was the suspected identity of Pink Panther; this was confirmed with presumptive testing followed by GC-MS analysis. One peak found during GC-MS analysis was not identified. Ivory wave was shown to contain Caffeine; this is suspected to be in a high concentration. The analysis of Blow determined that it contained four components, Phenyl acetic acid that is used in the manufacturing process of substituted amphetamines, 4-[3-(Dimethylamino)propoxy]benzaldehyde, which is used in chemical synthesis, Benzocaine, a suspected common adulterant of NPS and Methylphenidate, a class B stimulant.

Internal samples were then analysed to determine presence of the parent drugs, five of the compounds focussed on within this research are detected and this research suggests that actively feeding larvae are most likely to show chemicals ingested. Quantification was not attempted. It is possible that even though the samples were pooled, the concentration was still too low, this requires further investigation.

8.1 Summary

Post Mortem Interval (PMI) estimation envelops a great amount of forensic entomology research. The numerous factors recognized to effect these calculations are shown to potentially introduce error [8], [12], [15], [328]–[330], leading to an incorrect time of death prediction. One such acknowledged factor is the presence of drugs and toxins [22], [114], [128], [331], [332].

This research concentrates on one group of chemicals, Novel Psychoactive Substances, which is the focus of recent legislation 'Psychoactive Substances Act', 2016 [173], [175], [178], [182], [333]–[335], reacting to the increasingly widespread use and associated deaths. The aims of this study were firstly to determine an appropriate medium for delivery of these drugs, secondly to ascertain the developmental effects regarding forensically important blowfly and establishing the contingency of detection of such substances in blowfly larvae following ingestion, for the potential use of larval samples as alternative toxicological specimens. To conclude, cuticular hydrocarbon analysis [221], [232], [237], [238], [318] was utilised to demonstrate their potential for aging blowfly larvae affected by these chemicals.

Chapter 4 aimed to establish experimental protocols to allow the accurate completion of chapters 5-7. Chapter 5 determined the effect of eleven Novel Psychoactive Substances, three common adulterants and one illegal drug, all at two dosages, on the length, weight and development time of forensically important *C.vicina*. Comparison was also made between *L.sericata* for selected drugs. Chapter 7 showed the analysis of Novel

Psychoactive Substances (NPS) followed by investigation of larval internal samples for traces of NPS. Finally, Chapter 6 determined the feasibility of using cuticular hydrocarbons from blowfly larvae to accurately age specimens disregarding their apparent developmental state as a result of NPS ingestion.

8.2 Conclusions

Preliminary data presented in Chapter 4 covered a variety of topics, both related to the consequent chapters and also of interest for entomological research. Determination of the effects of sampling collection techniques on cuticular hydrocarbons enabled the use of pre-existing larval samples for this analysis. An artificial medium was developed to allow homogenous delivery of chemicals for experimental research; this was tested to determine its palatability and developmental effects resulting from nutritional contents. It was shown to be a suitable comparison in the absence of an animal model for drug testing. Further work also explained the development and definition of protocols for drug analysis and extraction of cuticular hydrocarbons.

Chapter 5 determined the drug induced changes on blowfly larvae physiology and consequently the prediction error concerning PMI. *C.vicina* was chosen as the focal species with comparisons made later in the chapter to *L.sericata*. Eleven NPS substances of unknown composition were researched alongside three common adulterants of both NPS and illegal compounds. Comparison of weights and lengths across the diets showed no difference on initial sampling days due to an insufficient exposure period. It was suggested that the organoleptic properties of the diet were influenced by the higher concentrations of drug added, deterring ingestion by the larvae. This is alleged due to the

development rate initially appearing slow, some populations were regarded as smaller than the control. Overall data showed acceleration of developmental rate with all drugs except paracetamol pupating 24-48 hours prior to the control. This would cause consequential error in PMI estimation. Paracetamol at a higher dosage was shown to delay pupariation by 48 hours. This study also concluded the importance of observing the effect of all components within a drug. Adulterants are prominent, and the three substances considered within this thesis, altered development substantially. Two dosages were considered for all drugs mentioned previously, with the results of four discussed. This highlighted the consequence of not only drug presence but the influence of concentration. The effect of dose was not uniform across all drugs. Caffeine was shown to increase acceleration of development with a higher concentration and paracetamol was shown to increase delay with a higher concentration. Substances Blow and AMT exhibited an acceleration of growth rate when combining doses, but separate analysis indicated the lower doses showed the most acceleration. It is hypothesized that the higher concentration influenced palatability of the diet, reducing intake. The effect of two drugs was compared on an alternative species, *L.sericata*, as previous research had suggested reactions of different species to drug presence could differ. The two drugs at two concentrations showed results correlating to those presented by *C.vicina*, it is not possible to say that developmental changes observed in *C.vicina* would all be applicable to *L.sericata* without the testing of further drugs. NPS are often based structurally on illegal drugs, the development effects of MDA were compared with the effects of 6APB to determine if studies concerning the influence of chemically similar illegal drugs could be utilised during PMI estimation. Based on the results shown when comparing 6APB and MDA, the use of MDA development data would provide accurate results for aging blowfly

larvae. This suggests the use of developmental data from chemically similar structures could be utilised for PMI estimation. The differences observed in drug availability and metabolites present post drug metabolism must be considered. This study considers the non-metabolised form of the named substances.

Chapter 6 shows analysis of the cuticular hydrocarbon profile across the larval stages. The profile was analysed after identification of cuticular hydrocarbons. The differences observed previously, due to the impact of the drugs, were not shown in hydrocarbon analysis. The results shown in this thesis suggest the hydrocarbon profile is not affected by the presence of drugs. Previous studies had shown the capabilities of the technique to differentiate between species and also to age specimens. This study encourages the use of cuticular hydrocarbon analysis for estimating age of larvae affected by drugs without the error potential shown in developmental data. The limitations of this research as a lab-based study are recognized and the need for field base research to incorporate external variables is acknowledged, together with further analysis to provide quantification of hydrocarbons to gain a complete understanding.

Chapter 7 focussed on the analysis of the NPS of focus within this research. Presumptive testing observations are presented alongside confirmatory GC-MS analysis. The composition of NPS is known to vary, it is however of interest within this study to determine an accurate analysis. Three batches of Benzofury were obtained and all three contained the suspected active ingredient, 6APB. It is believed due to abundance of the peaks shown in the chromatograms, that within Benzofury Green the active compound is in a lower concentration than the beige or blue batch. The substance labelled as 6APB was shown to be similar but not the same, a mass difference of 12 was assumed from the

fragment ions and the suspected chemical structure has been shown. 5EAPB was shown to contain the correct compound with the expected fragment ions observed. AMT, Synthacaine, Blow and Ivory Wave were shown to be a mixture of components. Internal analysis was carried out on larval samples pooled from each sampling day. Detection of the drug within the larvae both proves the ingestion of the substance by the larval samples and also enables them to be utilised as alternative toxicological samples at a crime scene. Detection was only possible for five of the drugs shown: Paracetamol, 6+5APB, 5EAPB, 'Benzofury Beige' and Benzocaine. All except for 5EAPB were only detected at one sampling point. 5EAPB was present within the internal samples of day five to day seven. The absence of drugs in other sample populations does not indicate drug absence from the food source; detection level, metabolism and elimination must all be considered.

Now that the NPS content is known, comparison of developmental effect between similar chemical compounds can be made. The Benzofury compounds tested were shown to contain the same active ingredient, 6APB with one displaying a lower concentration. 6+5APB was confirmed as either 6APB, 5APB or a mix of the two. 5EAPB was shown to be very chemically similar with only the addition of an ethyl group. The NPS purchased as 6APB was shown to be chemically similar also but with the addition of a carbon group. The populations tested on these drugs all pupariated prior to the control, between 24 and 48 hours. The Benzofury compounds along with 6+5APB all pupate at the same time with 6APB and 5EAPB pupate 24 hours later. It is not known if this is due to the additional groups shown during analysis or the effect of concentration. Although development appears to, on a whole show, a similar trend for these substances, it is obvious the effect

concentration alone can cause. After day four all populations are longer than the control and this continues until day seven when 6+5APB and Benzofury Green reach the post feeding stage and they shrink in length. See Figure 253 for larval length data, larval weight data is included in the appendix. Determination of composition and concentration prior to feeding trials could potentially enable the accurate comparison of these substances.

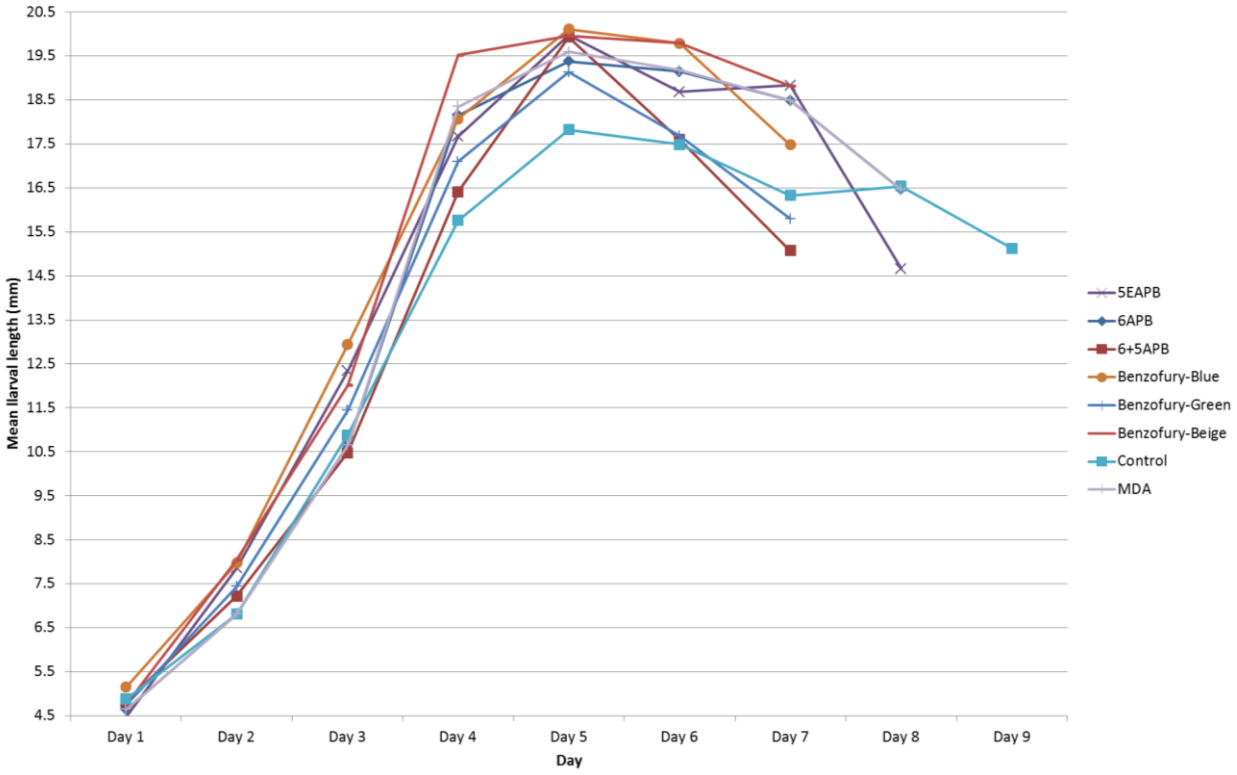


Figure 253: Length of larvae from similar APB drug diets across duration of development.

During confirmatory analysis Synthacaine was shown to contain six components, two of which were Caffeine and Benzocaine, concentrations were not measured. Comparison of the development of these drugs is shown in Figure 254 with weight data shown in the appendix. The development of Synthacaine is very similar to that of the Benzocaine population and pupariation occurs 48 hours prior to the control population in both of these studies. Caffeine samples show a quicker development rate; this could suggest that

the concentration of Caffeine is lower in Synthacaine as the effect on development is not as accelerated. The data shown in this research suggests that Synthacaine and Benzocaine are of similar composition.

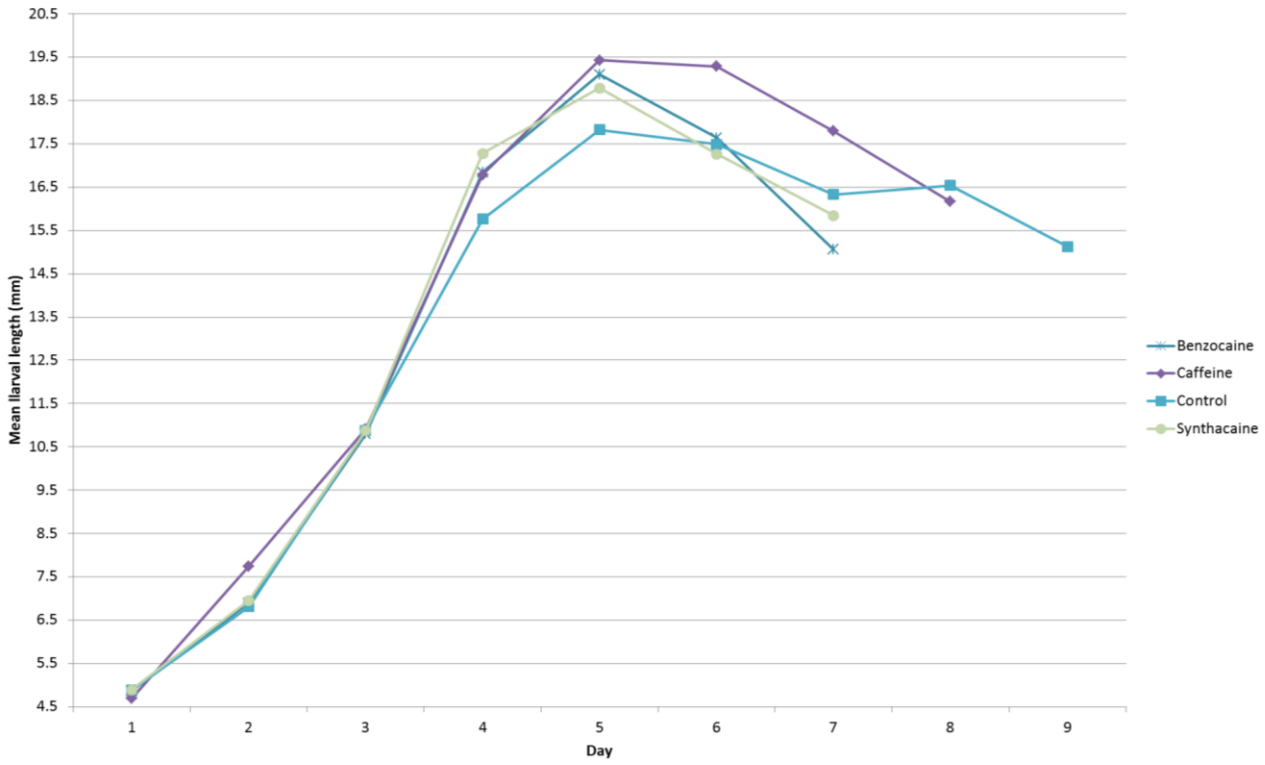


Figure 254: Length of larvae from drug diets similar to Synthacaine across duration of development.

GC-MS analysis concluded that the only compound identified within Ivory Wave was Caffeine. The comparison of the development of these two populations along with the control is shown in Figure 255, with weight development data shown in the appendix. It is suggested that the concentration of Caffeine within Ivory Wave is higher than that tested for pure Caffeine. Development is inhibited up to day three of sampling, potentially caused by unpalatability of the diet due to higher concentrations, then an increase in developmental rate is observed, reaching mean lengths above that shown within the Caffeine population and then pupariation occurs 48 hours prior to the control and 24 hours prior to the Caffeine samples.

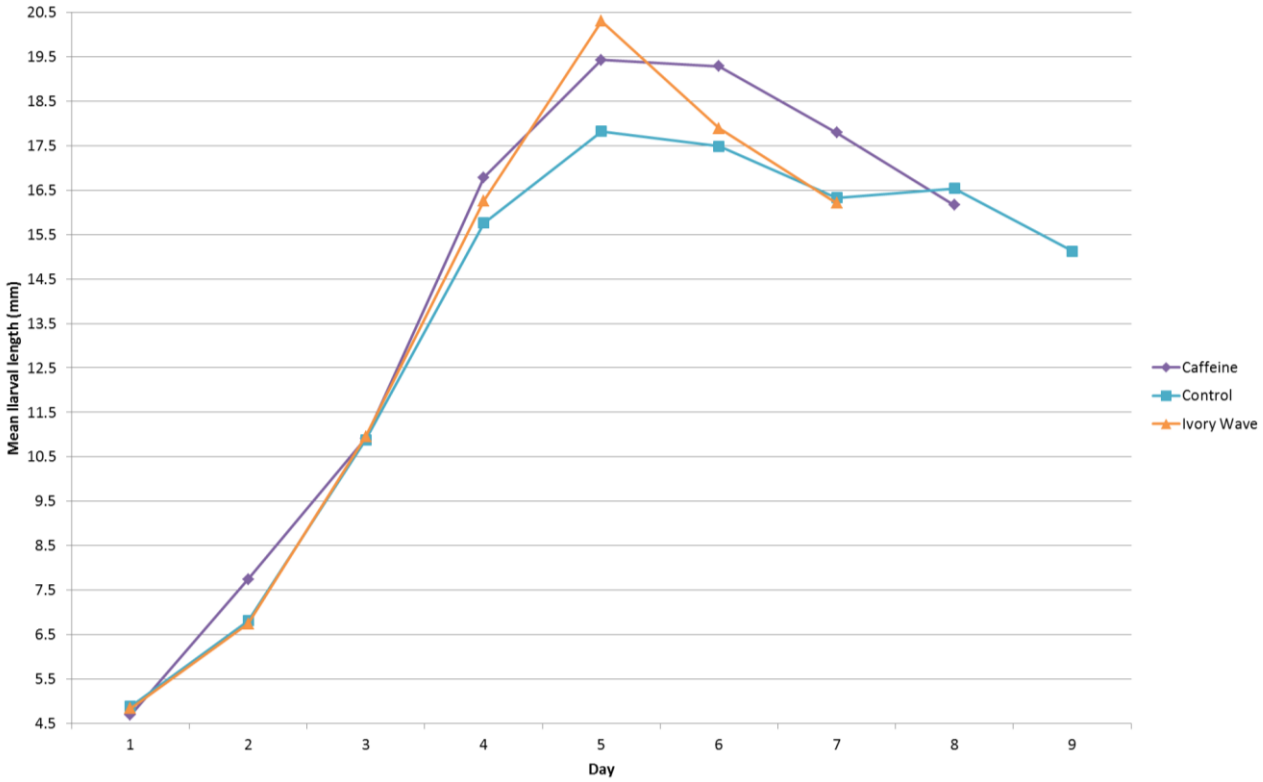


Figure 255 Line graph showing mean length of larvae from drug diets similar to Ivory Wave across duration of development.

8.3 Future Work

The next phase to follow this volume of research, leads in many directions. It would be of interest to determine the effect of concentration on the palatability of the larval diet. This was originally investigated within Chapter 4 with one drug concentration and no effect was observed. The studies following in Chapter 5 suggested that due to a perceived developmental delay, the appeal of the diet had been compromised. This was potentially owed to the larger drug presence. Further studies observing the changes to the amount of diet consumed and larval behaviour as a consequence of higher drug concentration would be applicable to our understanding of larval development when a drug is present.

Dosage was shown to be highly relevant regarding the rate of the effect on development. The effect of different dosages was shown not to be uniform across those drugs included, to expand this research to further concentrations both higher and lower to observe the trend would help to reduce PMI estimation error. It is also intended to extend this research to further blowfly species.

As a consequence of ethical concerns and also the inability to make use of drugged human tissue due to the Human Tissue Act, metabolism is the main limitation of this study. Further investigation into the differing effects on blowfly development of a drug and its metabolites would highly benefit the field of Entomotoxicology and the interpretation of experimental data carried out in this manner.

Cuticular hydrocarbon analysis showed potential for use of aging blowfly larvae when drugs have affected the physiology. Development of this analysis through quantification of the identified hydrocarbons could improve the results further. It would also be advantageous to determine the effect of external influences on cuticular hydrocarbons relating to field-testing. The hydrocarbons identified in the latter sampling days showed no real differences, this agrees with previous research [238]. The development however, is altered and the majority of specimens pupate early. This is not shown in the hydrocarbon profile and requires further investigation.

Finally, more work is intended to develop the method for internal analysis to provide more conclusive results as to drug presence. This will include determination of machine detection limits and further testing of the extraction protocol.

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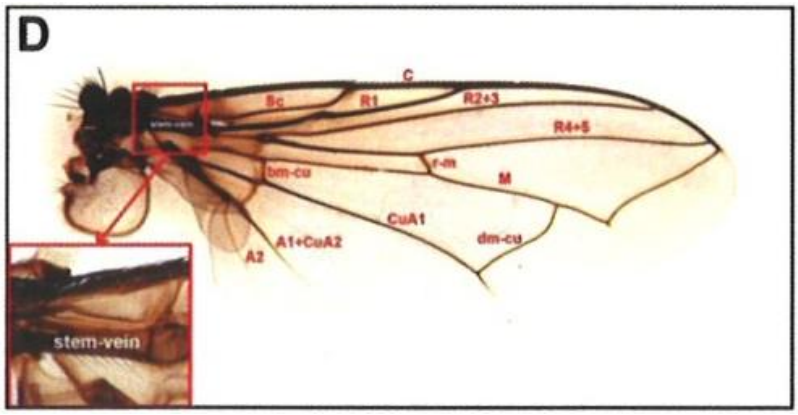
Appendix

Identification of *Lucilia sericata* blowfly adults:

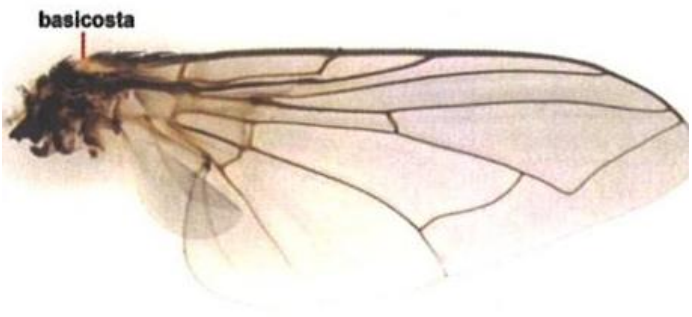
All images showing identification taken from [269] provided during an identification workshop.

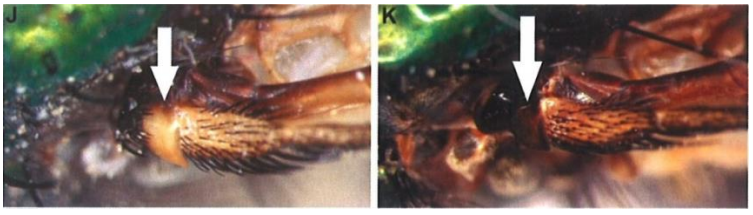
- **Stem vein is bare above**, as opposed to haired above.



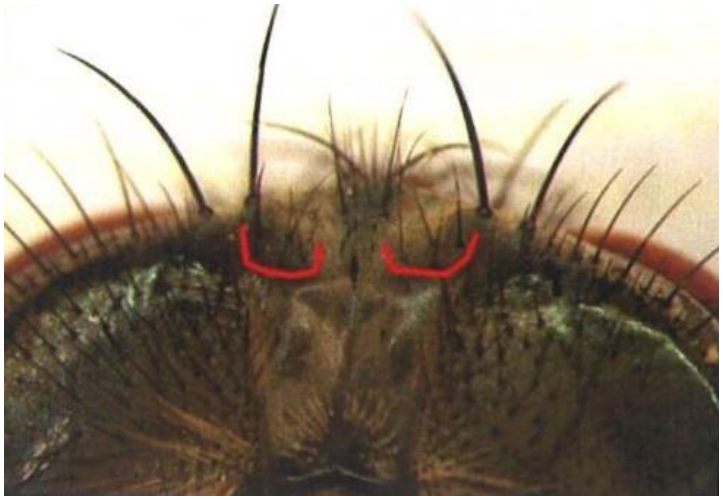
- **Thorax is bright metallic green** in colour, as opposed to dark and **lower calypter is bare above**.

- **The Basicosta is bright yellow** as opposed to dark.





- **The central occipital area has two to five bristles just below inner vertical bristle**. The abdomen is usually bright green/coppery in colour.

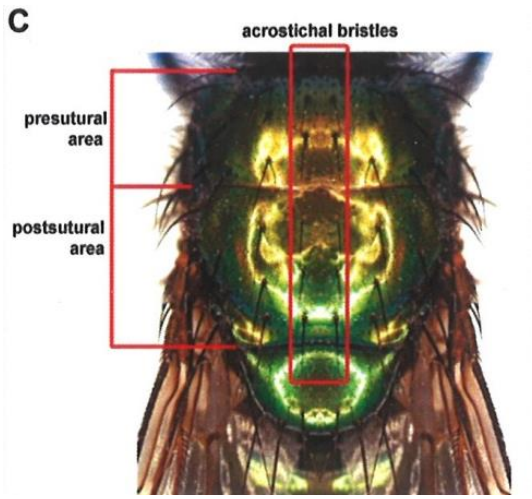


Identification of *Calliphora vicina* and *Calliphora vomitoria* blowfly adults

All images showing identification taken from [269] provided during an identification workshop.

- The **thorax is non-metallic and dark** in colour and the **lower calypter have hairs above**.

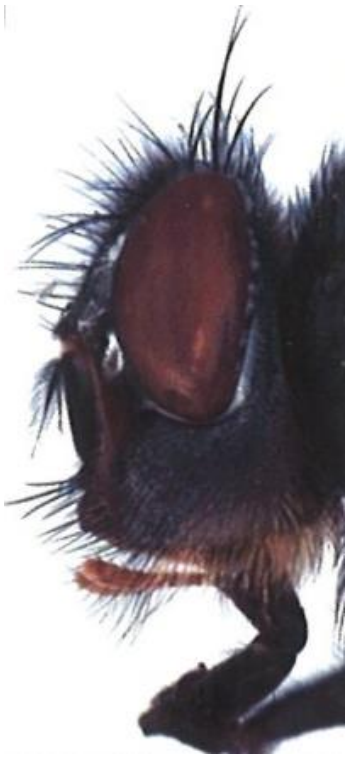
- **Three pairs of acrostichal bristles on postsutural area**. The **abdomen is shining blue but with weak microtrichosity**.



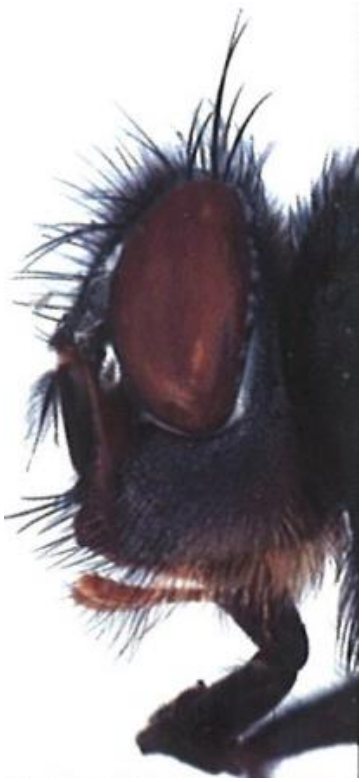
-**Upper and lower calypters are predominantly black**.

- ***Calliphora vicina*** – the **facial ridges, mouth edge and anterior part of genal dilation are orange**. **Basicosta is yellow**.

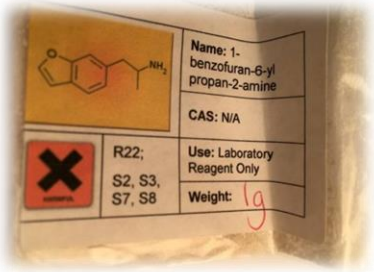
-***Calliphora vomitoria*** – **facial ridges, mouth edge and anterior part of genal dilation are dark** and **Basicosta is black**.

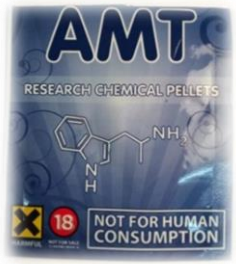


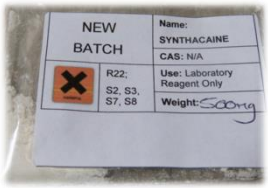
-***Calliphora vomitoria*****- Postgena and lower part of genal dilation have orange hairs.**

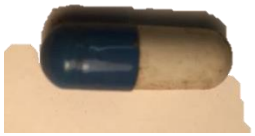
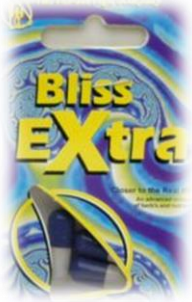
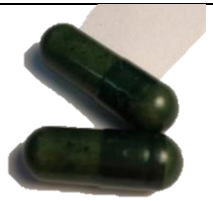


Sample Photo	Packaging Photo	Sample Name	Purchase Location
		6-(2-aminopropyl) benzofuran mixed with 5-(2-aminopropyl) benzofuran (6-APB with 5-APB).	OfficialBenzofury.com
		1-(benzofuran-5-yl)-N-ethylpropan-2-amine (5-EAPB)	OfficialBenzofury.com
		Blow	OfficialBenzofury.com

		Benzofury	OfficialBenzofury.com
		Benzofury	OfficialBenzofury.com
		6-(2-aminopropyl)benzofuran (6-APB)	ViP-Legals.com

		5-(2-aminopropyl)benzofuran (5-APB)	ViP-Legals.com
		Benzofury	ViP-Legals.com
		α-Methyltryptamine (AMT)	ViP-Legals.com

		Pink Panthers	legalhighsstore.co.uk.
		Synthacaine	legalhighsstore.co.uk.
		Ivory Wave	www.research-drugs.com.

		Pink Champagne	www.buckledbonzi.co.uk
		Bliss Extra	www.buckledbonzi.co.uk
		Druids Fantasy	www.buckledbonzi.co.uk

		L.S.A Hawaiian Baby Woodrose	www.herbalhighs.com
		Mitseez	www.herbalhighs.com
		Salvia (Blueberry)	www.offyourheadshop.com

		Salvia (Cherry)	www.midlandresearchchemicals.co.uk
		Benzocaine	eBay
		Caffeine	Acros Organics
		Paracetamol	Local Supermarket

Table 69: Photos and purchase location of Novel Psychoactive Substances used during this research.

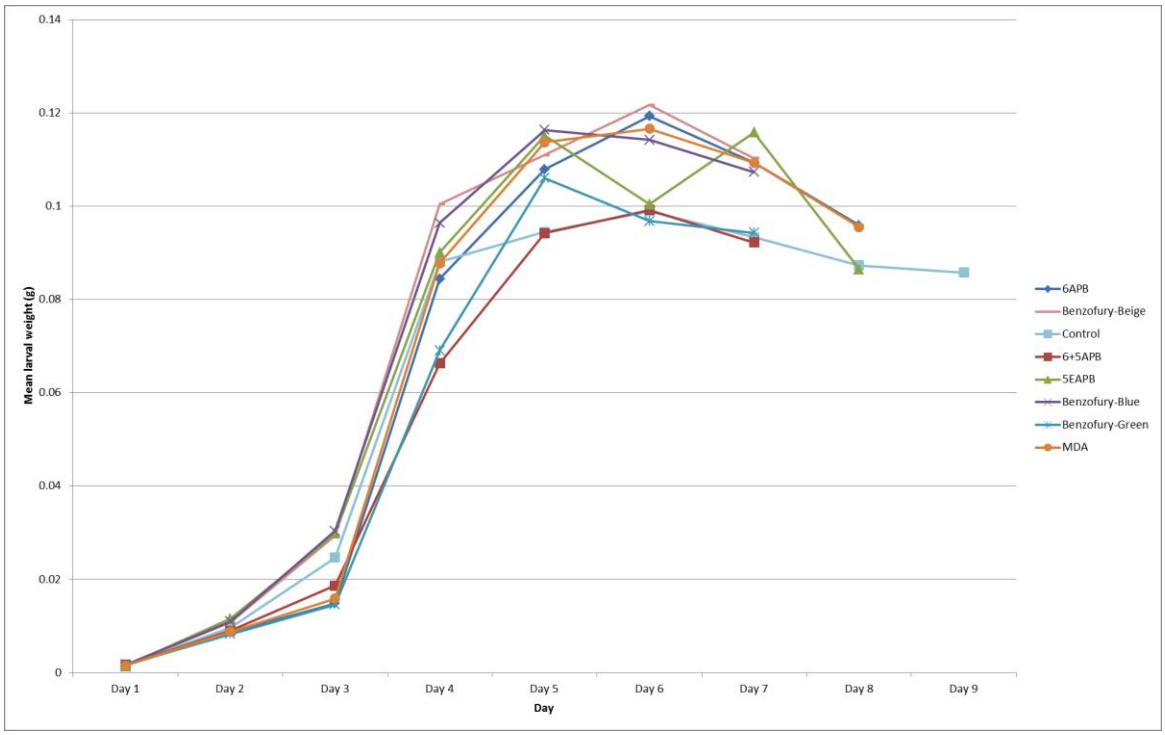


Figure 256: Line graph showing mean weight of larvae from similar APB drug diets across duration of development.

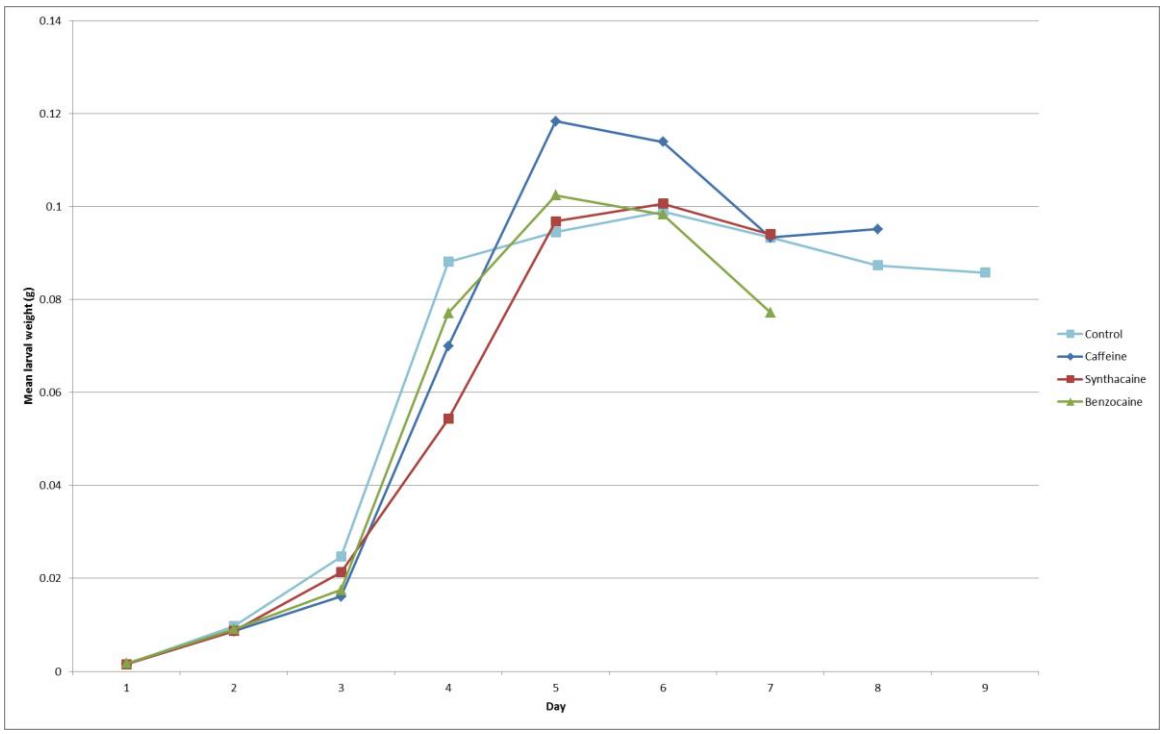


Figure 257: Line graph showing mean weight of larvae from drug diets similar to Synthacaine across duration of development.

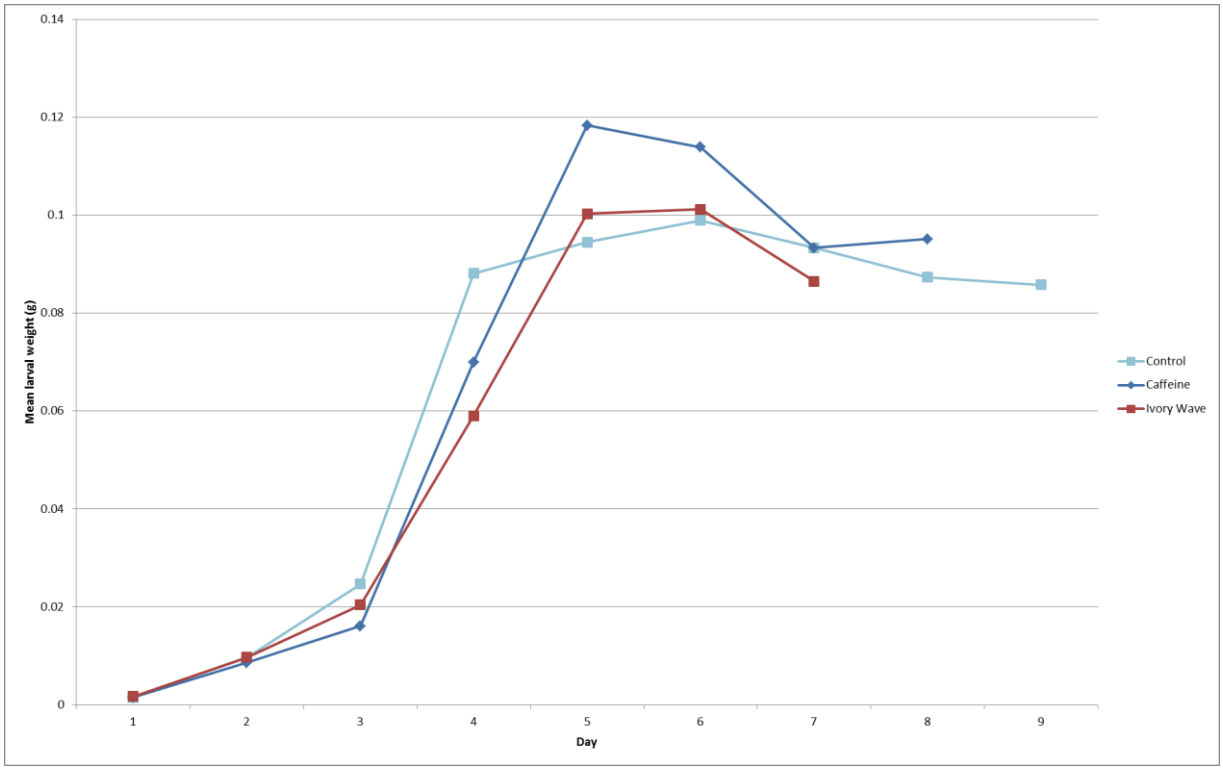


Figure 258: Line graph showing mean weight of larvae from drug diets similar to Ivory Wave across duration of development.

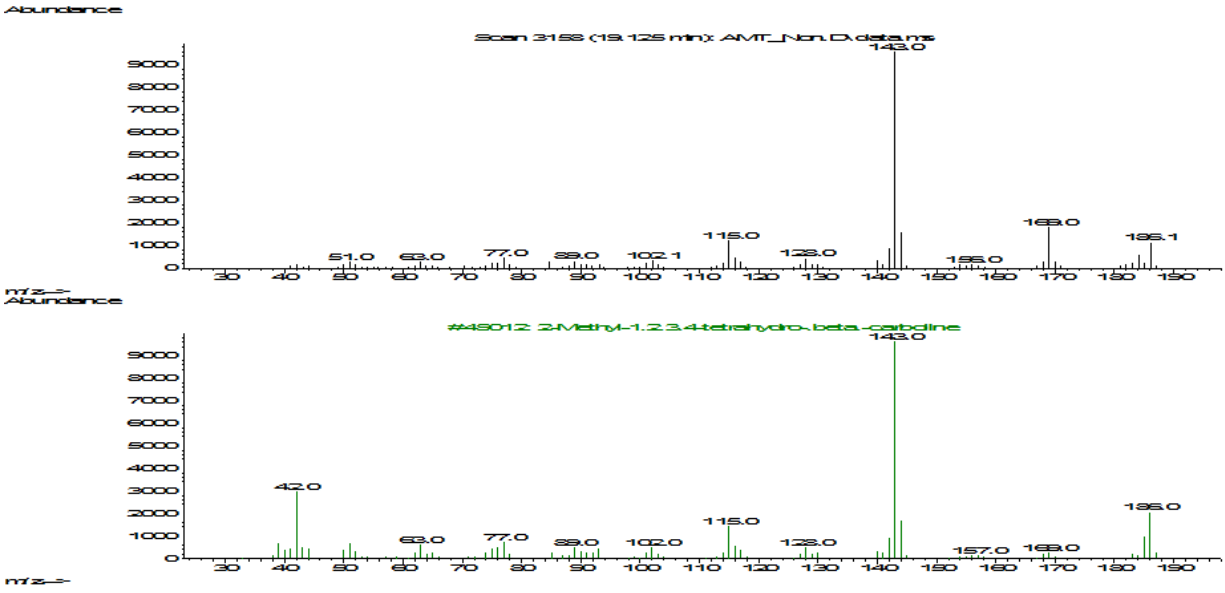


Figure 259: AMT -19.1min -NIST Library identification.

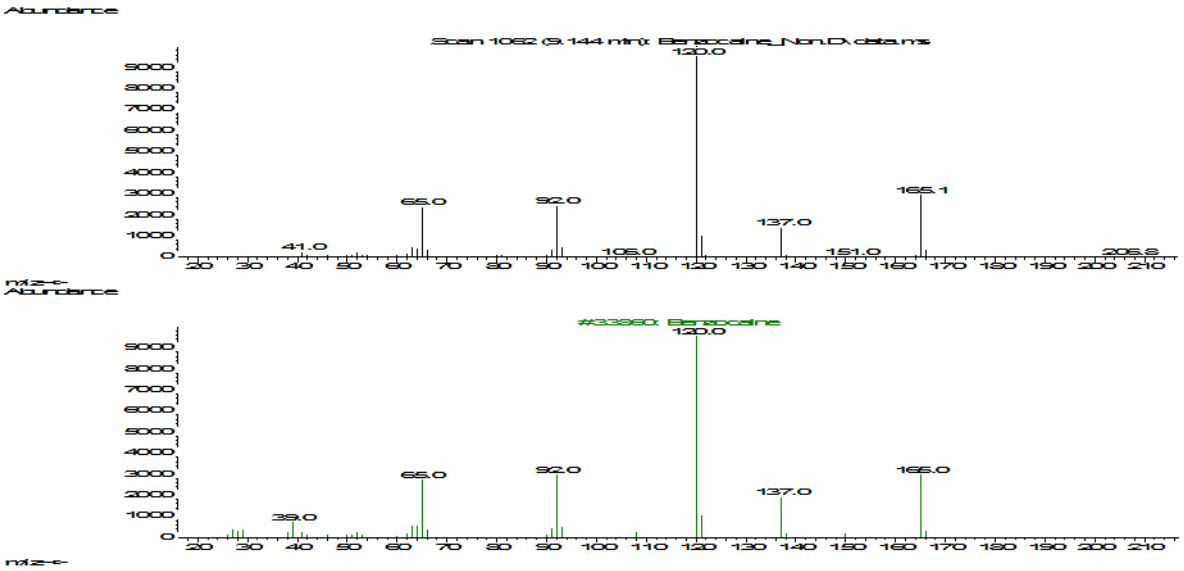


Figure 260: Benzocaine -9.1min -NIST Library identification.

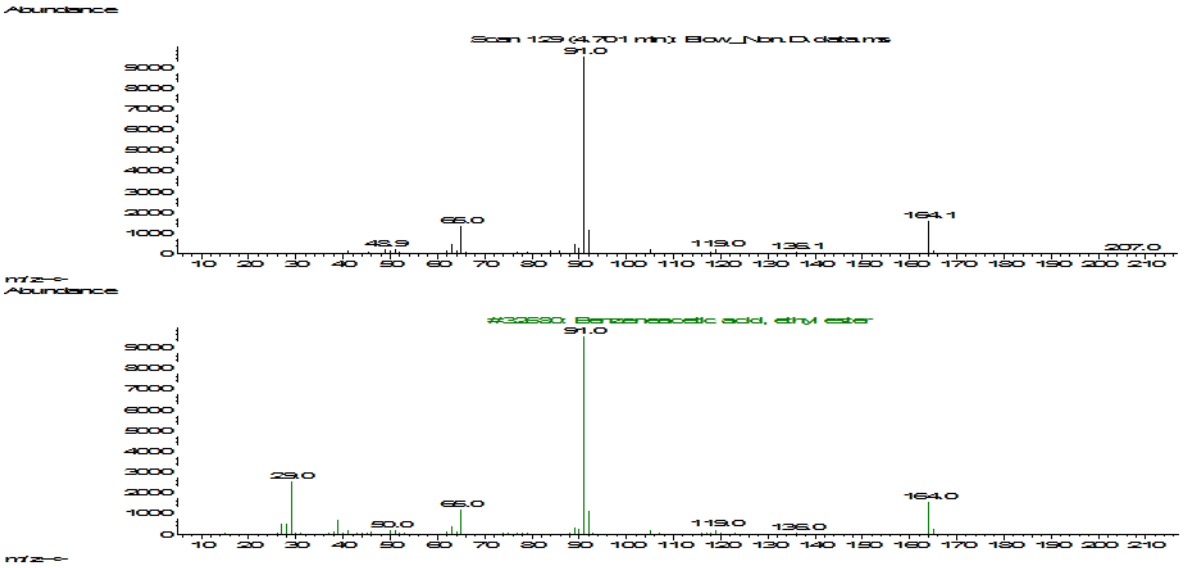


Figure 261: Blow - 4.7min -NIST Library identification.

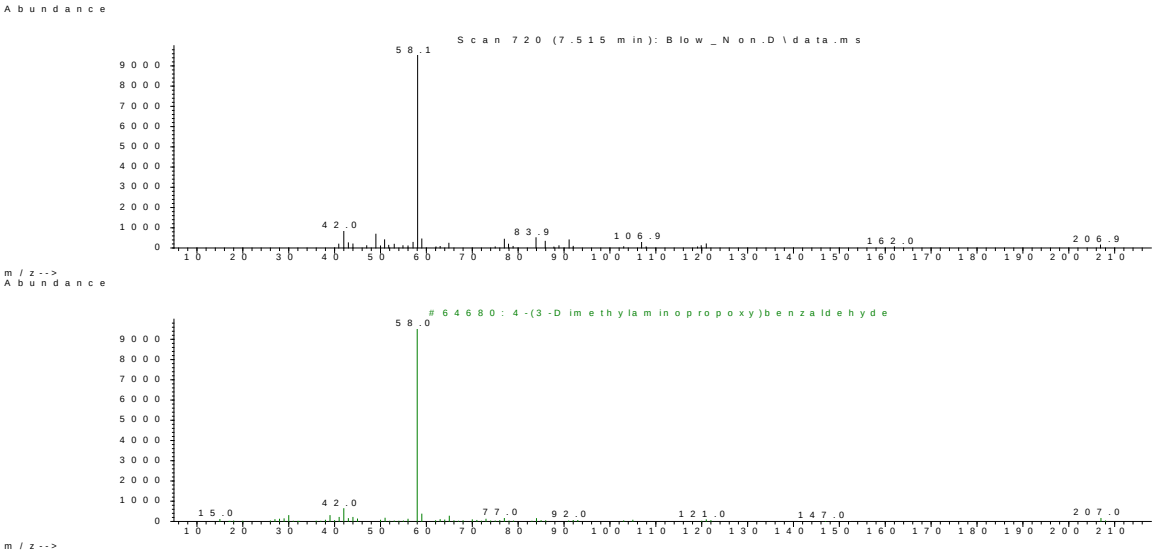


Figure 262: Blow – 7.5min -NIST Library identification.

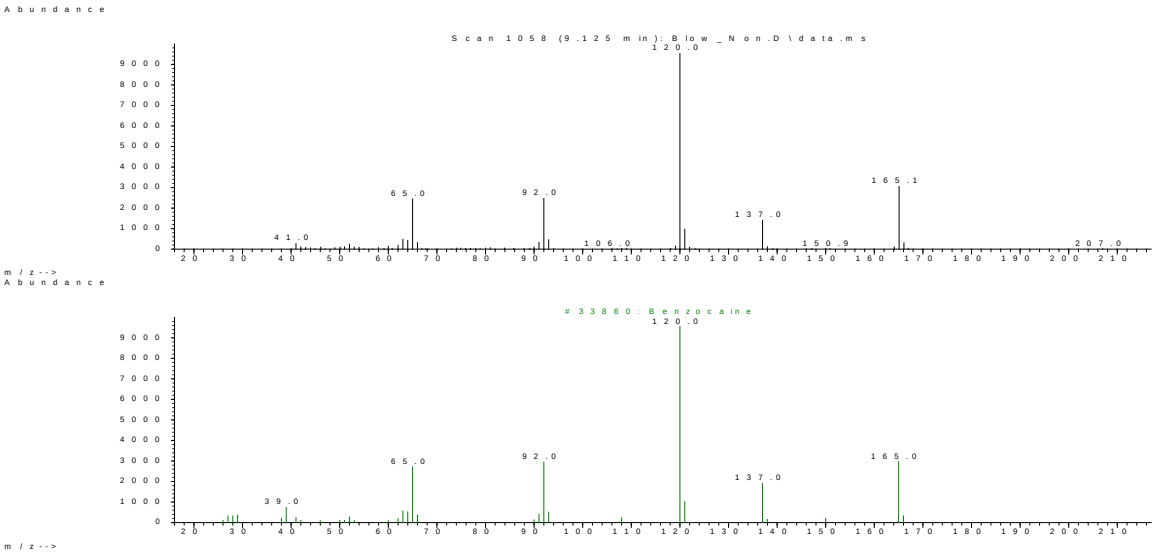


Figure 263: Blow – 9.1 min -NIST Library identification.

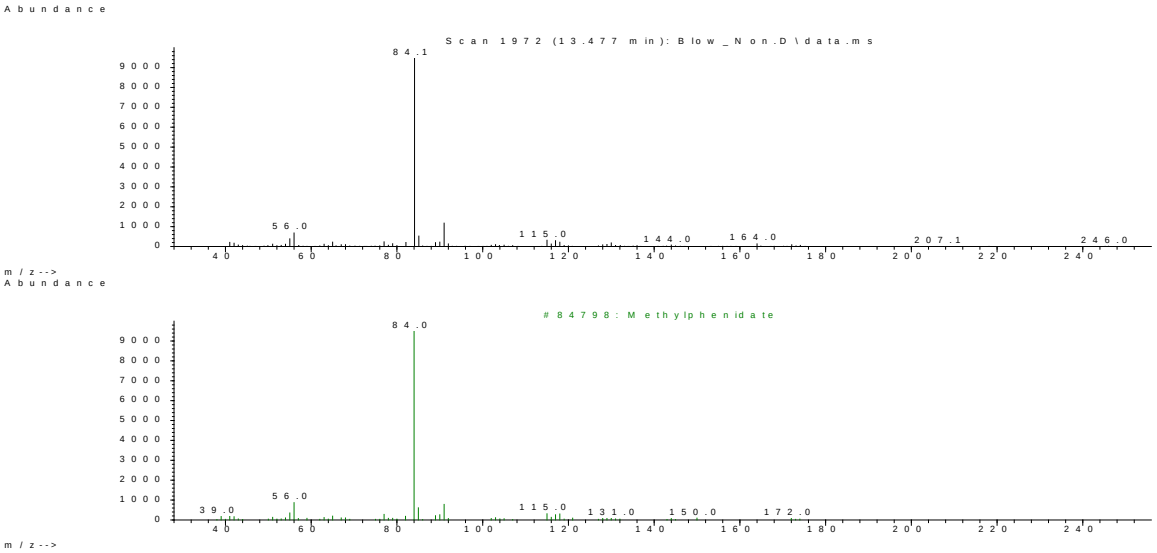


Figure 264: Blow – 13.4 min -NIST Library identification.

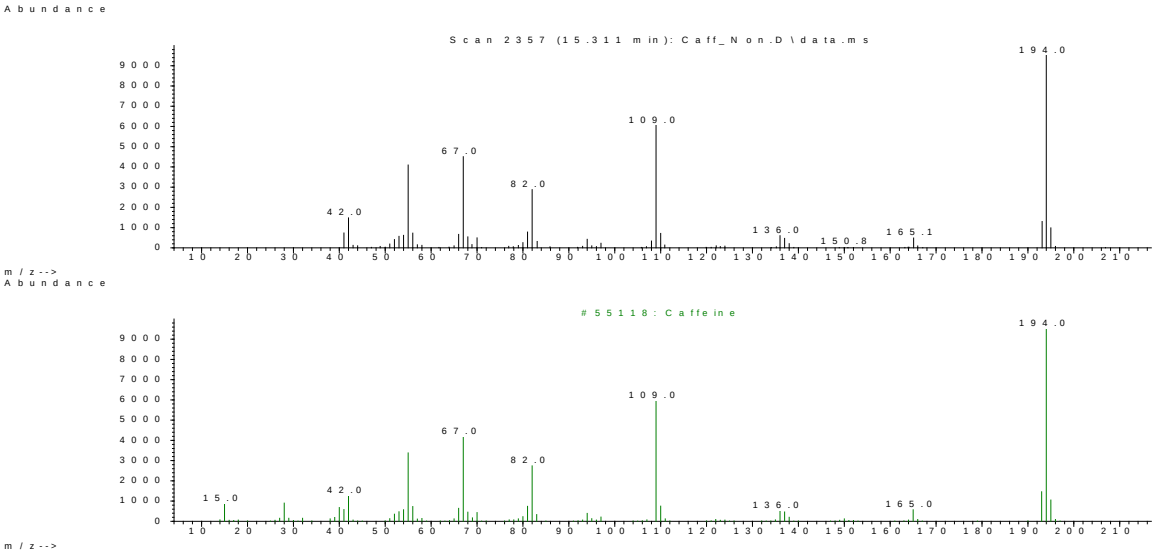


Figure 265: Caffeine – 15.3 min -NIST Library identification.

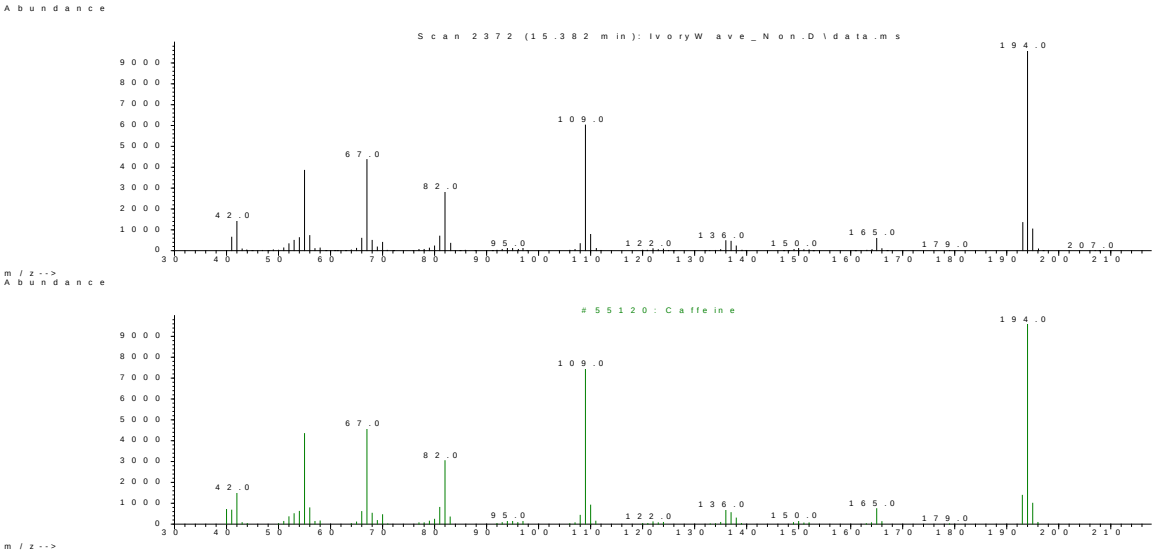


Figure 266: Ivory Wave – 15.3 min -NIST Library identification.

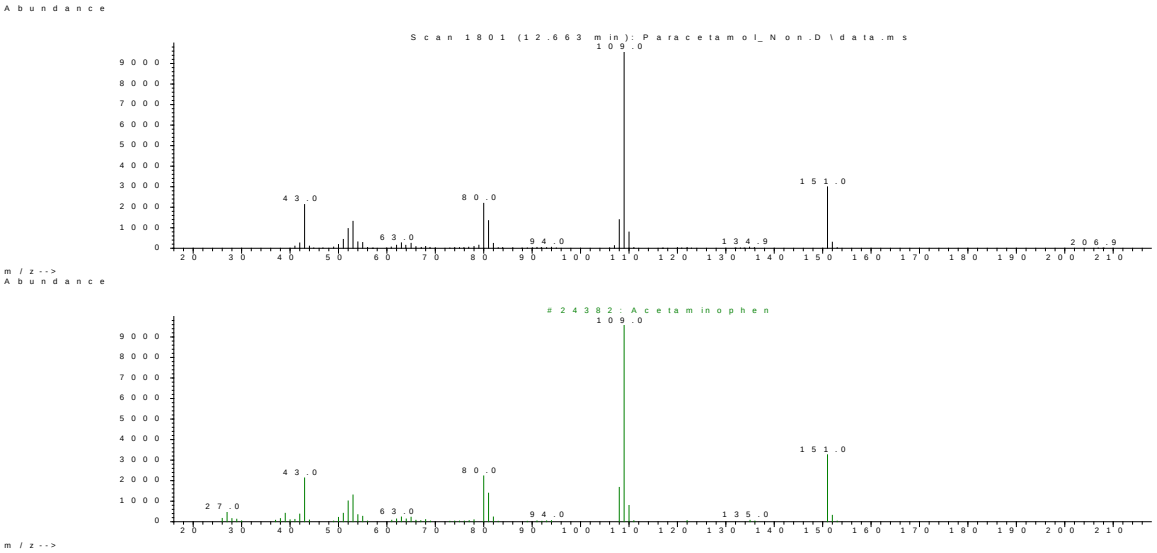


Figure 267: Paracetamol – 12.6 min -NIST Library identification.

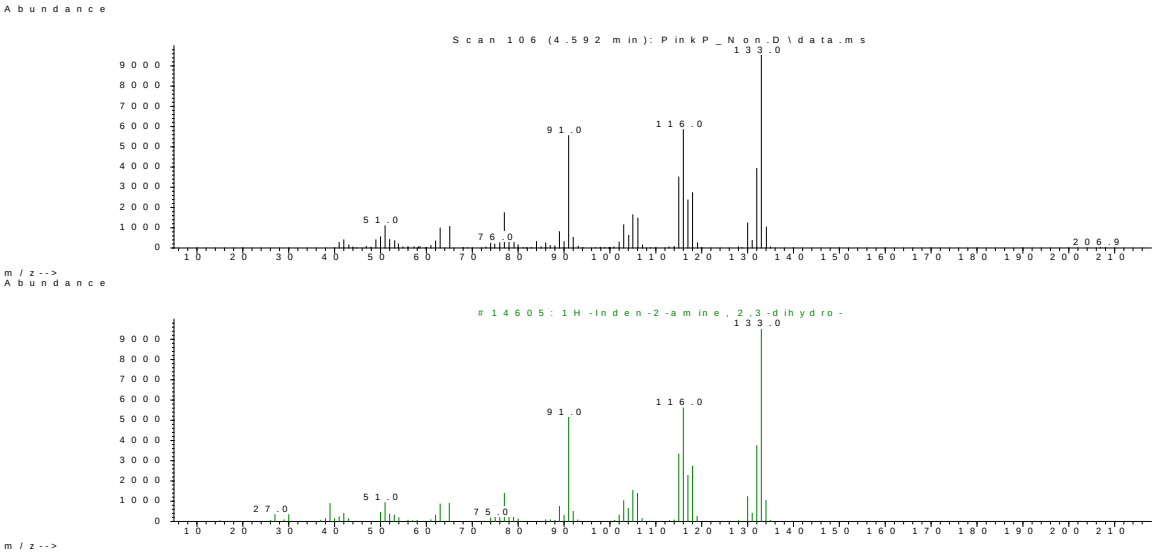


Figure 268: Caffeine – 4.5 min -NIST Library identification.

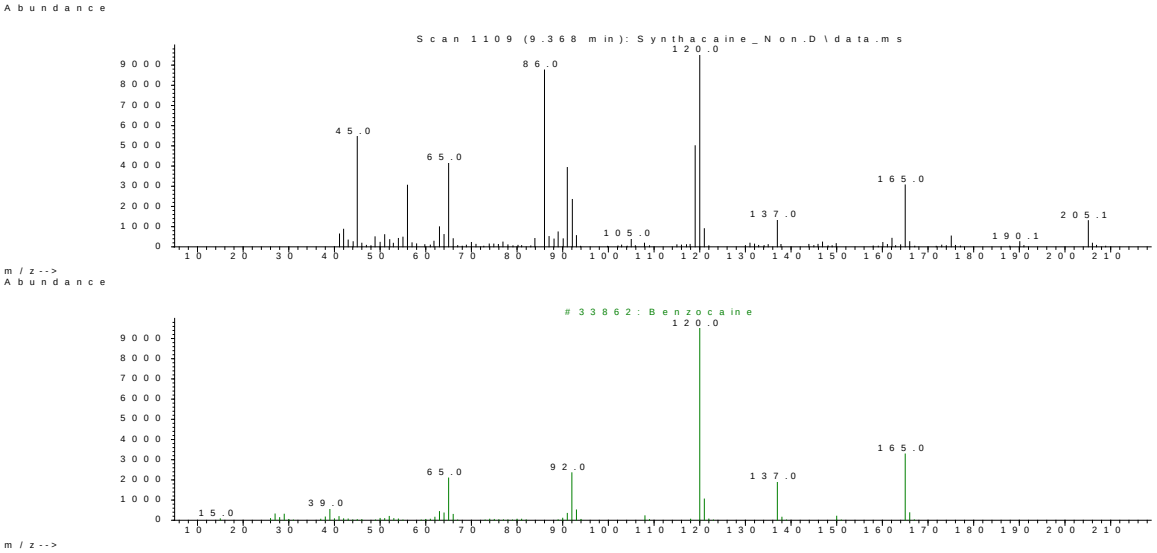


Figure 269: Synthacaine – 9.3 min -NIST Library identification.

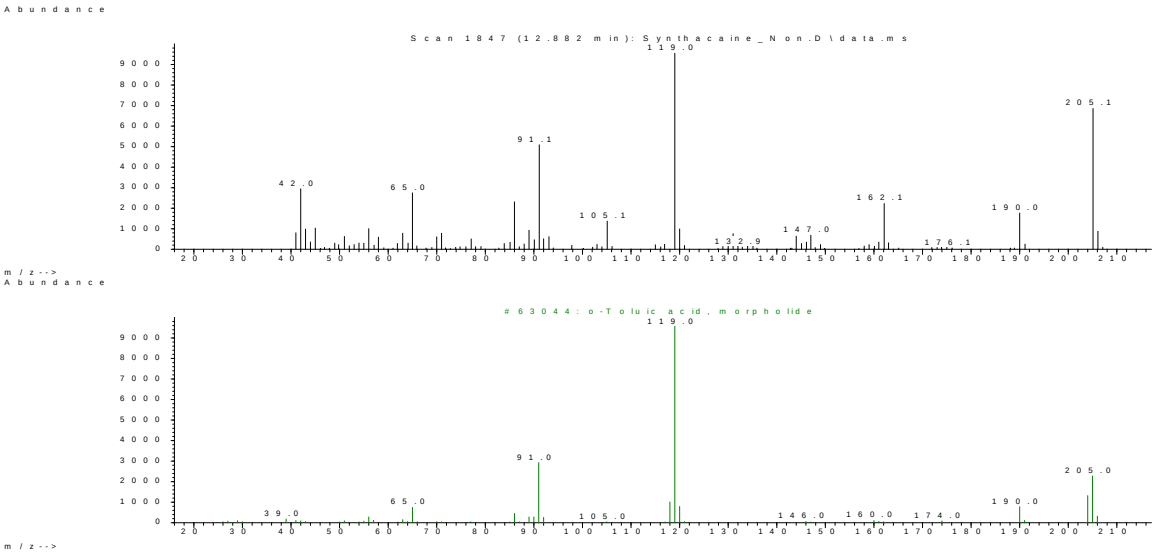


Figure 270: Synthacaine – 12.8 min -NIST Library identification.

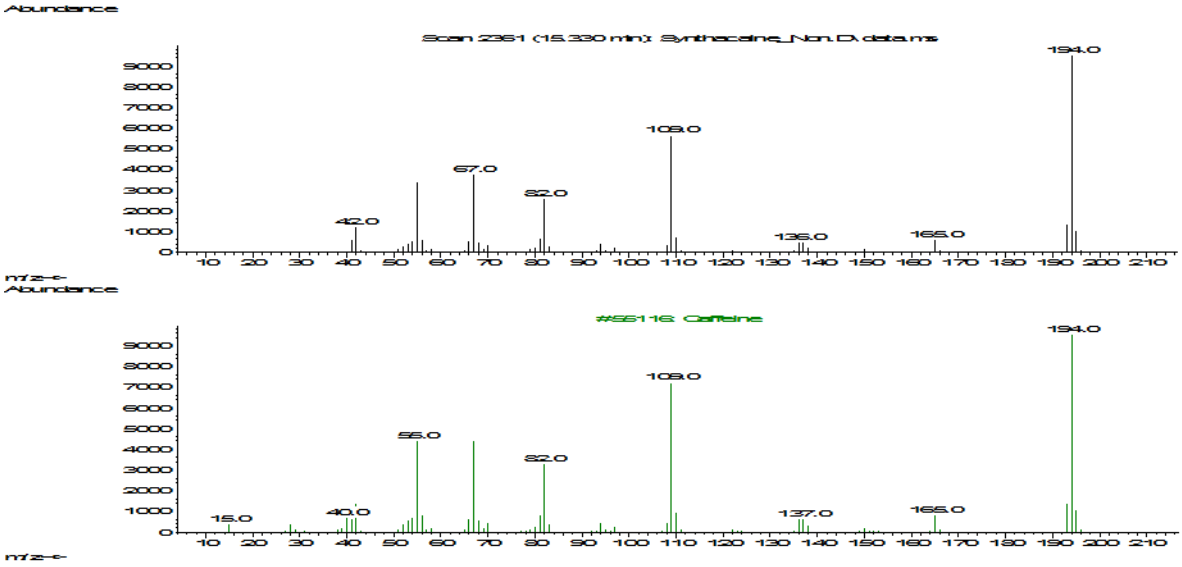


Figure 271: Synthacaine – 15.3 min -NIST Library identification.

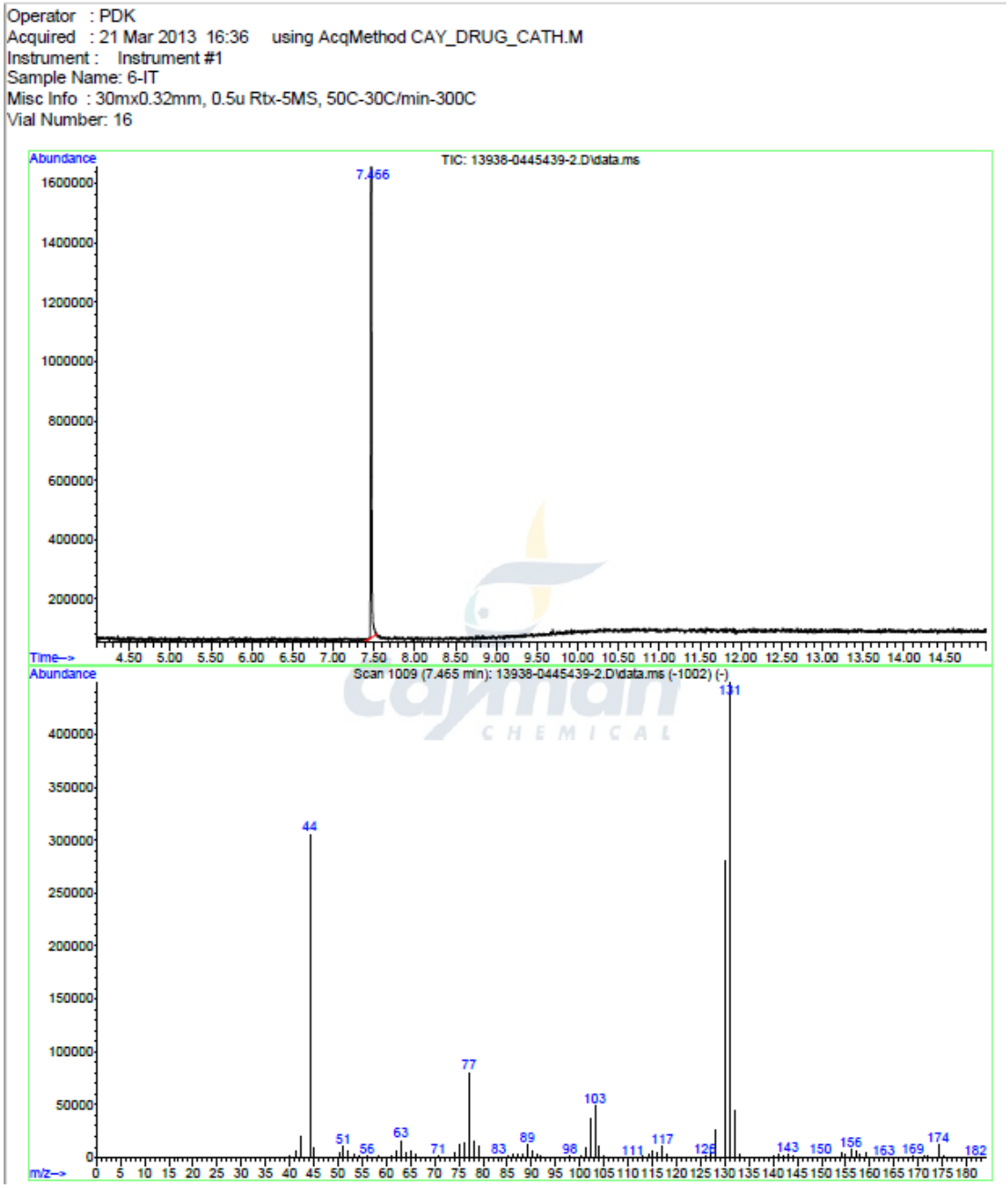


Figure 272: 6IT reference material [336].

le :Y:\Forensics\11079-0440572.D
perator : PDK
cquired : 30 Aug 2012 12:45 using AcqMethod CAY_DRUG_CATH.M
strument : Instrument #1
ample Name: 6-APB
isc Info : 30mx0.32mm, 0.5u Rtx-5MS, 50C-30C/min-300C
al Number: 16

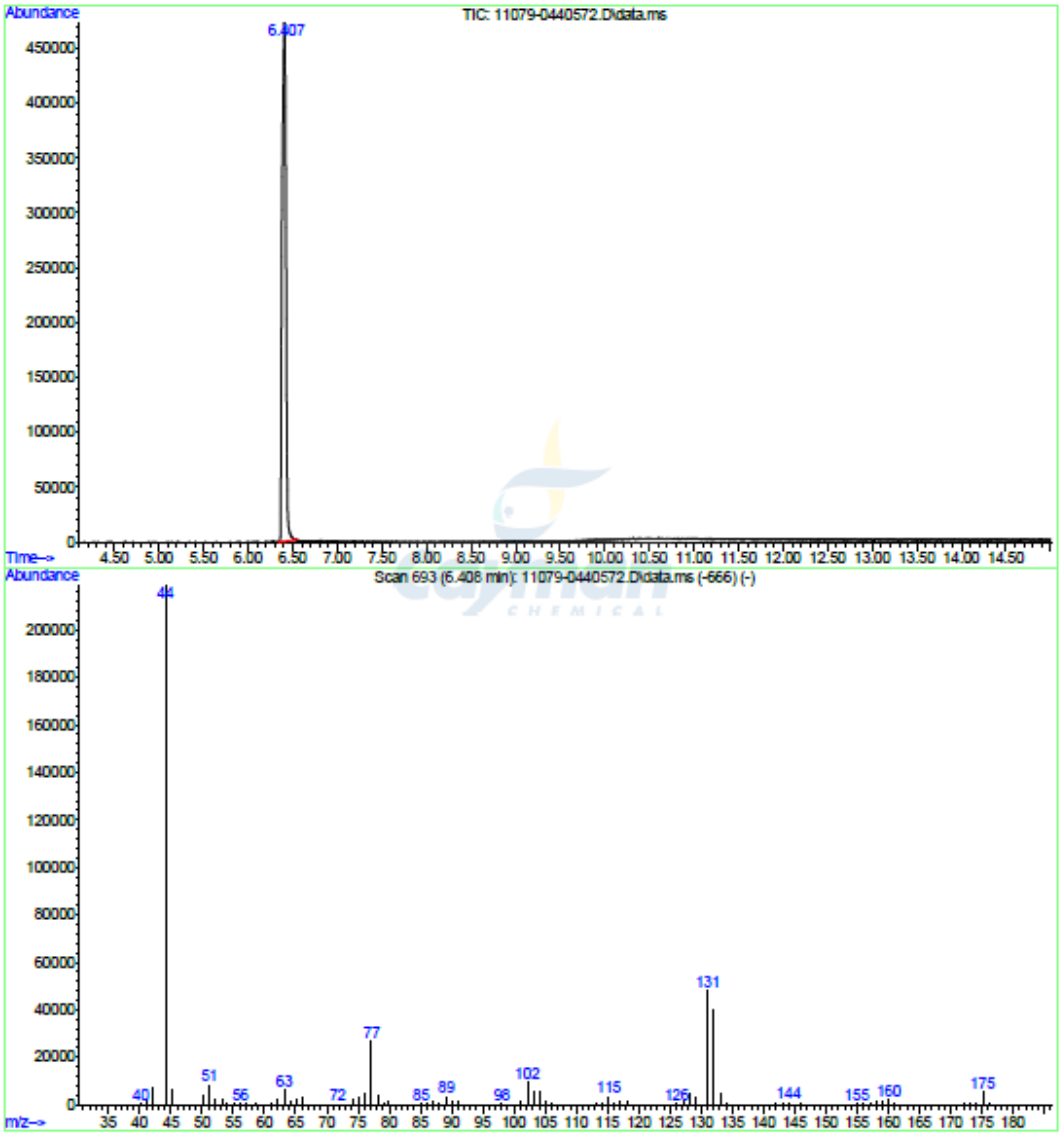


Figure 273: 6APB reference material [336].

ile :Y:\Forensics\11161-0439618-3.D
)perator : PDK
cquired : 3 Aug 2012 15:09 using AcqMethod CAY_DRUG_CATH.M
nstrument : Instrument #1
ample Name: Methiopropamine
lisc Info : 30mx0.32mm, 0.5u Rtx-5MS, 50C-300C/min-300C
ial Number: 2

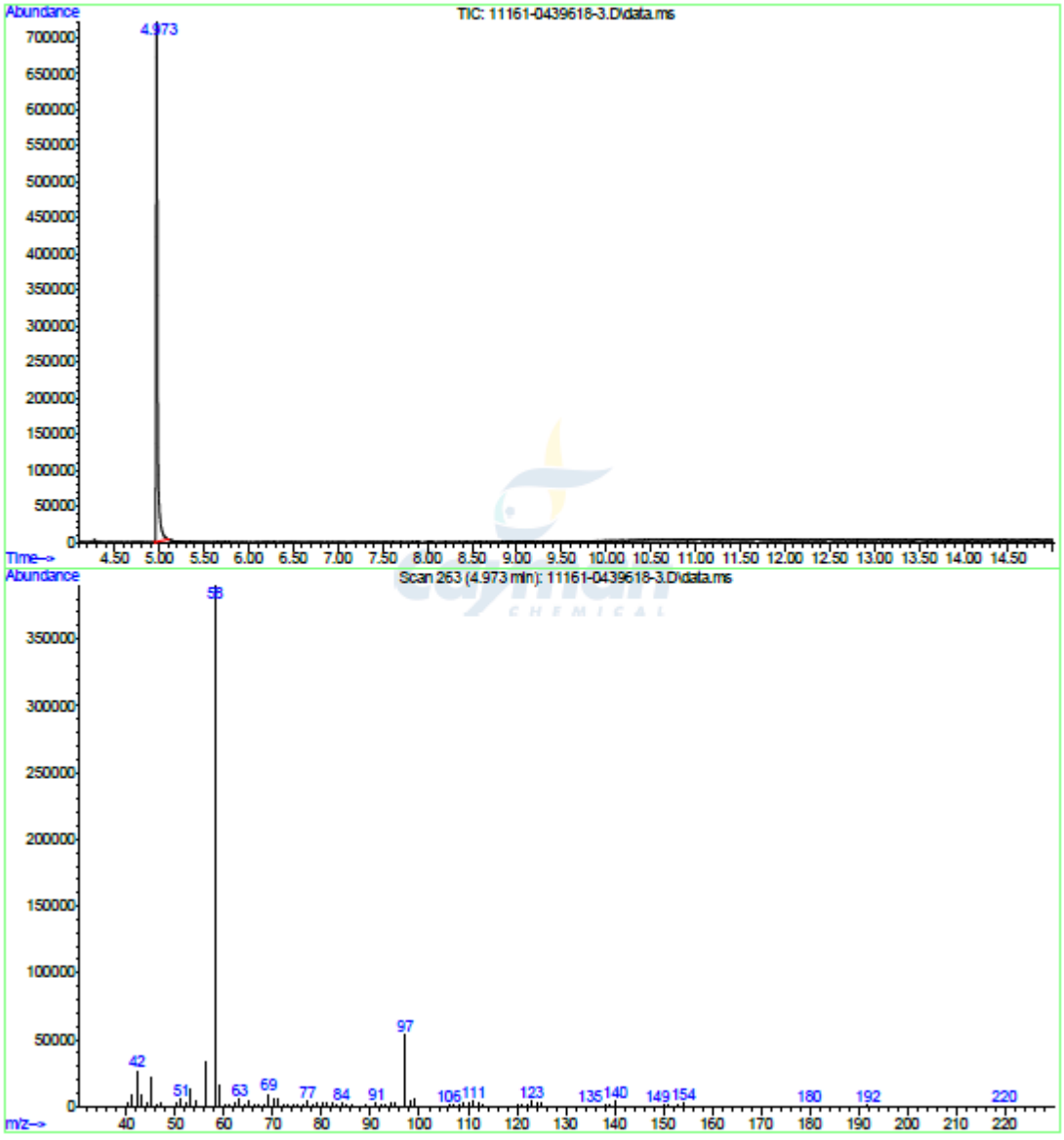
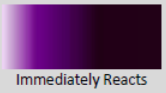
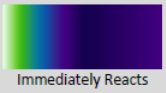
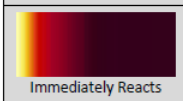
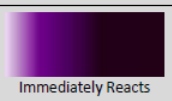
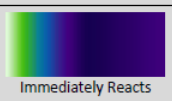
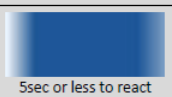
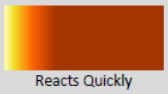
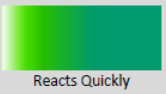
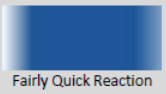
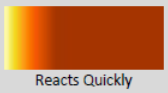
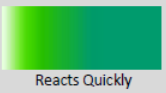
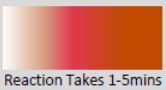
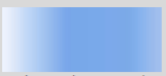
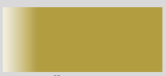
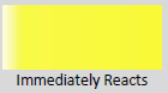
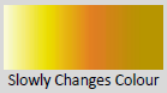
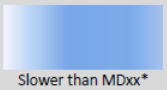
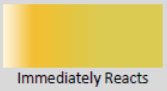
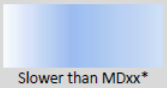
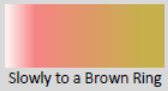
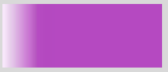
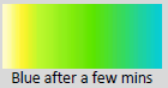
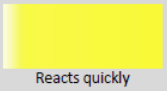
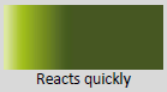
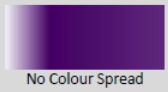


Figure 274: MPA reference material [336].

MARQUIS	MANDELIN	MECKE	SIMON'S	ROBATEST	SUSPECTED DRUG
 5sec or Less to React	 Immediately Reacts	 Immediately Reacts	 5sec or Less to React	***No Reaction***	**MDMA or MDEA**
 5sec or Less to React	 Immediately Reacts	 Immediately Reacts	***No Reaction***	 Reaction takes 1-5mins	**MDA**
 Immediately Reacts	 Immediately Reacts	 Immediately Reacts	 5sec or less to react	***No Reaction***	**MDMA or MDEA & Methamphetamine**
 Reacts Quickly	 Reacts Quickly	***No Reaction***	 Fairly Quick Reaction	***No Reaction***	**Methamphetamine**
 Reacts Quickly	 Reacts Quickly	***No Reaction***	***No Reaction***	 Reaction Takes 1-5mins	**Amphetamine**
Immediately Reacts	 Immediately Reacts	 Slowly Changes Colour	 Slower than MDxx*	 Small Brown Ring	**Methylone (bk-MDMA)**

 Immediately Reacts	 Immediately Reacts	 Slowly Changes Colour	 Slower than MDxx*	 Small Brown Ring	**Methylone (bk-MDMA)**
No Reaction (Some impure batches have a yellow reaction)	 Immediately Reacts	***No Reaction*** (may smoke)	 Slower than MDxx*	 Slowly to a Brown Ring	**Mephedrone (4-MMC)**
	***Unknown*** (Likely no reaction)	***Unknown*** (Possibly green) reaction	***Unknown***	***Unknown***	**LSD**
No Reaction	 No reaction with 1yr old kit! USE NEW KIT!	***No Reaction*** (Pale light yellow after 5min with very new kit)	***No Reaction***	***No Reaction***	**Ketamine**
			***No Reaction***	***No Reaction***	**Opiates (such as Codeine / Morphine / Heroin)**
 Blue after a few mins	 Reacts quickly	 Reacts quickly	***No Reaction***	 No Colour Spread	**2C-B**
No Reaction	Fairly Quick Reaction	Fairly Quick Reaction	***No Reaction***	No Colour Spread	**2C-T-7**
>10sec to react	Slow to Green	Immediately Reacts	***No Reaction***	***No Reaction***	**DXM**

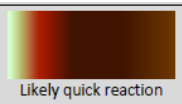
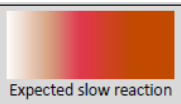
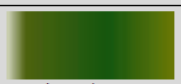
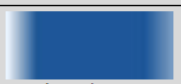
No Reaction	 Likely quick reaction	 Sometimes no reaction	***No Reaction***	 Expected slow reaction	**PMA**
No Reaction	 Fairly quick reaction	 Fairly quick reaction	 Fairly quick reaction	***No Reaction***	**PMMA**
No Reaction	***No Reaction***	***No Reaction***	***No Reaction*** (or may have a faint blue reaction)	***No Reaction***	**Piperazines (such as BZP / TFMPP / mCPP)**
No Reaction		***No Reaction***	***Unknown***	***Unknown***	**Strychnine**

Figure 275: Potential colour combinations following presumptive drug testing [337].